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CELLULAR MECHANISMS OF ADAPTIVE IMMUNE RECONSTITUTION FOLLOWING HUMAN BONE MARROW TRANSPLANTATION

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Abstract

Successful immune reconstitution decreases morbidity and mortality following human bone marrow transplantation (BMT). Unmanipulated haploidentical BMT followed by post-transplant cyclophosphamide (pt-Cy) has recently been introduced not only to provide a donor for nearly all patients lacking an HLA-matched donor, but also to improve immune competence through the transfer of adaptive immune cells with the graft. However, the mechanisms of reconstitution and the Cy-effect on transferred immune cells remain unknown. Here, we investigated the dynamics of B and T cell reconstitution in this setting and provide evidence that they follow two different mechanisms. Adoptively-transferred mature B cells fail to persist in the recipient and the recovering B cells derive from a *de novo* differentiation process from haematopoietic stem cells or B cell precursors. They display an immature/transitional phenotype in the early weeks post-transplant and then progress through four differentiation stages identified by CD5 and CD21 expression, including a novel CD5⁺CD21⁺ stage of transitional differentiation. Mature, naïve B cells later replace transitional cells and predominate in the B cell compartment for 6 months. In contrast, transferred naïve T cells (T_N) preferentially survive Cy compared to memory cells. T_N rapidly differentiate into T memory stem cells endowed with superior reconstitution capacity within days post-infusion and later contribute to peripheral reconstitution by differentiating into effectors. Similarly, pathogen-specific memory T cells generated detectable recall responses but only in the presence of the cognate antigen. Therefore, T cell immunity is at least in part preserved following pt-Cy and may act as a first line of defence against reactivating pathogens and residual tumor cells. Differently, B cell immunity is generated *de novo* and takes at least 6 months to phenotypically resemble that of healthy donors.

Overall, these findings define the cellular basis of adaptive immune reconstitution process and shed light on basic aspects of T and B cell differentiation and persistence.

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1 Introduction

1.1 Bone marrow and haematopoietic cell transplantation

Bone marrow transplantation (BMT) and haematopoietic stem cell transplantation (HSCT), following either marrow-ablative or non-myeloablative conditioning regimen, are widely used for the treatment of a variety of hematological malignancies including lymphoma, acute and chronic leukemias, multiple myeloma, myelodysplastic syndrome and myeloproliferative disorders¹. This therapy comprises the administration of a transplantation conditioning regimen, which consists of high-dose chemotherapy with or without total body irradiation, followed by the intravenous transfusion of marrow or peripheral blood (PB) stem cells from the donor. Human leukocyte antigen (HLA)-matched sibling donors are generally chosen for these procedures because they are best associated with overall and progression-free survival². However, donor availability represents a major problem as only about one-third of candidates for allogeneic BMT (allo-BMT) have HLA-matched siblings³. When HLA-matched sibling donors are not available, there are three possible source of haematopoietic stem cells: HLA-matched unrelated donors (who are not always identified in the international registries), umbilical cord blood and partially HLA-mismatched, haploidentical, related donors, who have one HLA haplotype in common with the recipient⁴. Haploidentical donors can be identified rapidly in nearly all cases because the patient shares one HLA haplotype with each biological parent or child and half of his/her siblings. However, graft rejection and graft-versus-host disease (GVHD) remain the major complications of HLA-haploidentical BMT (haploBMT)⁵⁻⁸. These are both consequences of the diversity in the histocompatibility complex antigens⁹ and result

from the excessive alloreactivity of host T cells¹⁰, in the case of graft rejection, and of donor T cells¹¹, in the case of GVHD.

Ex-vivo T cell depletion, which removes T cells from the donor graft, may be employed to prevent GVHD without the morbidity associated with immunosuppressive drugs¹²⁻¹⁶. This procedure significantly reduces the incidence and severity of GVHD, but does not improve overall survival. Indeed, donor T cells in the recipient play a major role in the prevention of infections and mediate malignancy eradication (graft-versus-tumor effect)¹⁷. Thus, the absence of donor T cells in the graft is associated with disease relapse and a high risk of serious infection and death due to prolonged immune deficiency in the recipients¹⁸⁻²³. For these reasons, other methods are required to selectively inhibit alloreactivity, while preserving immunity against pathogens and tumor cells.

1.2 Unmanipulated HLA haploBMT with high dose cyclophosphamide

A non-myeloablative conditioning regimen followed by non-T cell-depleted (unmanipulated) BMT from an HLA-haploidentical donor has recently been proposed in patients with poor risk hematological malignancies and non-malignant hematological disorders^{3,24-27}. The key feature of this clinical protocol, first applied by a group from Baltimore^{3,24,25}, is the employment of high dose, post-transplant cyclophosphamide (pt-Cy) given to prevent GVHD. According to studies in mice²⁸, Cy selectively depletes alloreactive T cells *in vivo* after infusion of unmanipulated grafts and spares bystander naïve and memory T cells^{3,25,29}.

Cy is a highly immunosuppressive antineoplastic agent that has been employed since the 1960s to modulate the allograft response³⁰. It causes apoptosis in proliferating cells and, in the allo-BMT context, would be expected to deplete

allogeneic antigen-stimulated cells and cause clonal deletion. Typically, the drug was administered before transplantation to prevent graft rejection by suppressing the host immune system. However, it has been demonstrated that pre-transplantation conditioning regimen with Cy increases the risk of GVHD following allogeneic T cell infusion in mouse models³¹. On the other hand, the administration of a properly timed, high dose of Cy after BMT inhibited both graft rejection and GVHD^{28,32-34}.

Given that T cells undergoing replicative DNA synthesis are uniquely sensitive to Cy, both anti-host and anti-donor T cells are selectively destroyed²⁹. In the non-T cell-depleted haploBMT protocol used by the Baltimore group, allogeneic T cells are activated quickly after transfer and are therefore depleted by the administration of Cy^{25,29}. Non-allogeneic T cells are spared and can subsequently mediate immune reconstitution in response to the lymphopenic environment of the patient.

The Baltimore group protocol gives good results in terms of clinical outcome: engraftment occurs rather rapidly and non-relapse mortality, acute and chronic GVHD and survival are comparable to other studies where reduced conditioning regimen and HLA-haploidentical donors were employed^{3,35}. The most important result, however, was that serious opportunistic infections were diminished. Indeed, deaths from infection were low despite the incidence of reactivation^{25,26,29,36}. Using the same clinical protocol, Raiola et al. reported that the incidence of cytomegalovirus (CMV) reactivation was 42% and that EBV DNAemia was detected in 23% of patients. However, no patient died of CMV disease, invasive fungal infection or sepsis and no post-transplant EBV-associated lymphoproliferative disorders were observed²⁶, suggesting effective immune reconstitution in these patients.

1.3 Immune reconstitution after haploBMT

The immune reconstitution process dictates clinical outcome in patients who receive transplantation. Independently of the clinical protocol, all patients undergoing BMT inevitably experience ablation of the immune system following high-dose chemotherapy administered before transplantation. Conditioning of the patients, depending on its intensity, ablates or strongly constricts host haematopoiesis to suppress the patient's immune system, with the aim of preventing graft rejection. As a consequence, the transplant preparative regimen results in a severe and protracted lymphopenia, which is followed by the immune reconstitution phase.

Recovery of a fully functional immune system is a slow process following allogeneic stem cell transplantation and its duration differs between the various immune cell populations. The innate immune system, which includes neutrophils, monocytes, dendritic cells (DCs), macrophages and natural killer (NK) cells, normalizes rapidly compared to the adaptive immune system³⁷. It is documented that neutrophils reconstitute at a median of approximately 15 days after unmanipulated haploBMT³, whereas NK cells circulate at normal levels by 1 month post-transplant³⁸⁻⁴³. In contrast, DCs recovery is delayed and lies chronologically between innate and adaptive immune recovery^{40,41,44-51}. While innate immunity reconstitutes quickly, adaptive B and T cell recovery is delayed for years following transplantation, resulting in a global immune deficiency³⁷. The following section will be focused on adaptive immune cells and will describe the T and B cell compartments and their immune reconstitution process.

1.4 Adaptive immunity: T cell compartment

T lymphocytes belong to the adaptive arm of the immune system. They derive from progenitors that arise from multipotent haematopoietic stem cells in the bone marrow (BM) and subsequently migrate to the thymus to undergo a process of maturation that includes the rearrangement of the T cell receptor (TCR) genes. TCRs mediate the antigen recognition through interactions with linear peptides presented by MHC molecules on the surface of antigen-presenting cells (APCs) or target cells⁵². TCR consists of a variable TCR $\alpha\beta$ heterodimer that forms a multimeric receptor complex with the CD3 complex, which exists as a series of dimers including $\gamma\epsilon$, $\delta\epsilon$, and $\zeta\zeta$ associated with a single $\alpha\beta$ heterodimer^{53,54}. The binding of TCR $\alpha\beta$ with complexes of peptide and MHC molecules results in biochemical changes in the cytoplasmic portions of the CD3 complex like the phosphorylation of the tyrosine-based activation motifs (ITAMs) by the Src family tyrosine kinases LCK and FYN, essential for TCR signalling⁵⁵.

TCR $\alpha\beta$ heterodimers are generated during thymic development: thymocytes recombine variable (V), diversity (D) and joining (J) segments of the locus encoding TCR β , and V and J segments of the locus encoding TCR α , to form functional receptors. Diversity is further increased through the addition and subtraction of nucleotides at the gene segment junctions^{56,57}. This method of generating receptor diversity provides TCR repertoire which contains an estimated 2.5×10^8 different TCRs in the periphery of humans⁵⁸, which enables highly specific immune responses to a diverse range of foreign antigens⁵².

TCR rearrangements is followed by the so-called positive and negative selection⁵⁹. Positive selection is the process through which the thymocytes become restricted by the host major histocompatibility complex (MHC): only thymocytes able to bind the host MHC with sufficient affinity, able to initiate an immune

response, will survive and will be released in the periphery⁵⁹. In contrast, the process of negative selection deletes thymocytes that recognize self peptides in the context of host MHC molecules with high affinity. This mechanism ensures that only thymocytes recognizing non-self peptides are released in the periphery. It is estimated that 75% of thymocytes are eliminated by negative selection in the thymus⁶⁰. Post-thymus mature T cells, called naïve T cells (T_N), retain low levels of self-reactivity that are insufficient to induce autoimmunity, but essential for survival, as the interaction with self peptide-MHC (pMHC) complexes regulates their homeostasis^{61,62}.

T_N leave the thymus and migrate through the blood and lymph to secondary lymphoid tissues, i.e. the spleen and lymph nodes, where they continuously scan APCs in search of cognate non-self antigens⁶³. If cognate antigen is encountered, T_N undergo a process of activation characterized by extensive proliferation (clonal expansion) and acquisition of effector functions that are necessary to eliminate infected cells⁶⁴. When the pathogen is cleared, about 95% of T cells die, but a small fraction survives, giving rise to a long-lived population termed memory T cells (T_{MEM})⁶⁵. These cells are clonally expanded and ensure more rapid recall activation than naïve cells, thus providing protection following re-infection.

1.4.1 Heterogeneity of the T cell population

The memory compartment comprises T cell subsets that differ in terms of phenotypic composition, functional activity and capacity to respond to homeostatic or antigenic simulation⁶⁶. Indeed, the T cell compartment is highly heterogeneous. In 1999, Sallusto and Lanzavecchia reported that antigen-experienced $CD4^+$ and $CD8^+$ T cells can be divided into two main subsets, termed central memory (T_{CM}) and effector memory (T_{EM}), on the basis of their migratory capacity in the body. The

more differentiated T_{EM} preferentially migrate to peripheral tissues and show immediately effector capacities in response to antigen re-exposure⁶⁶. On the other hand, the less differentiated T_{CM} localize to secondary lymphoid organs and show little effector functions, although they have a high capacity to proliferate and differentiate into effector cells in response to antigen stimulation⁶⁷⁻⁶⁹.

T_{CM} are thought to maintain long-term memory in a stem cell-like fashion. Recently, studies in mice and later in humans and rhesus macaque, identified a new memory T cell population, termed T stem cell memory (T_{SCM})⁷⁰⁻⁷². These cells, which represent the 2-3% of all circulating CD4⁺ and CD8⁺ human T cells, exhibit features between naïve and memory cells⁷¹. Indeed, T_{SCM} display a largely T_N-like phenotype, retain a core of genes expressed by T_N and, similar to T_N, have a tropism for secondary lymphoid tissues⁷¹⁻⁷³. Conversely, T_{SCM} have low TREC content and have functional capacities of conventional memory T cells: like T_{CM} and T_{EM}, T_{SCM} undergo proliferation in response to interleukin (IL)-15 and, upon superantigen stimulation, rapidly acquire effector functions producing IL-2, TNF- α and interferon (IFN)- γ ⁷¹. Collectively, these data demonstrate that T_{SCM} constitute an intermediate population between naïve and memory cells, and represent the earliest differentiated memory T cell population in humans⁷¹⁻⁷³.

T_{SCM} also retain stem cell-like qualities, including a higher capacity to self-renew *in vitro* and to generate more differentiated progeny compared to T_{CM}^{71,73}. Indeed, after anti-CD3/CD2/CD28 stimulation, which induces T cell differentiation, T_{SCM} demonstrate a twofold higher capacity to maintain the original phenotype when compared with T_{CM}⁷¹. Moreover, T_{SCM} display greater multipotency compared to all the other memory cells as demonstrated by their ability to generate all other memory subsets, including T_{CM}^{71,73}.

Between T_{CM} and T_{EM}, further T cell subset has been characterized, termed transitional memory (T_{TM})^{74,75}, which appears to be more differentiated than T_{CM} but not as fully differentiated as T_{EM} in terms of phenotype^{76,77} and magnitude of expansion in response to IL-15^{78,79}. In addition, a T terminal effector (T_{TE}) subset comprises the most differentiated T cells^{74,75}. They have low proliferative and functional capacity^{75,80,81}, tend to undergo apoptosis and have the shortest telomeres among T cells⁸², thus indicating terminal differentiation^{74,75}.

These observations suggest that memory T cell differentiation in humans progresses linearly according to the relationship T_N→ T_{SCM}→T_{CM}→T_{TM}→T_{EM}→T_{TE}^{71,74,75} (Figure 1). In this model, T cells lose some abilities, such as the capacity to migrate to secondary lymphoid tissues, multipotency and self-renewal (*i.e.*, the capacity to persist in the body), but gain others, such as effector functions and tropism for peripheral tissues⁷¹⁻⁷³.

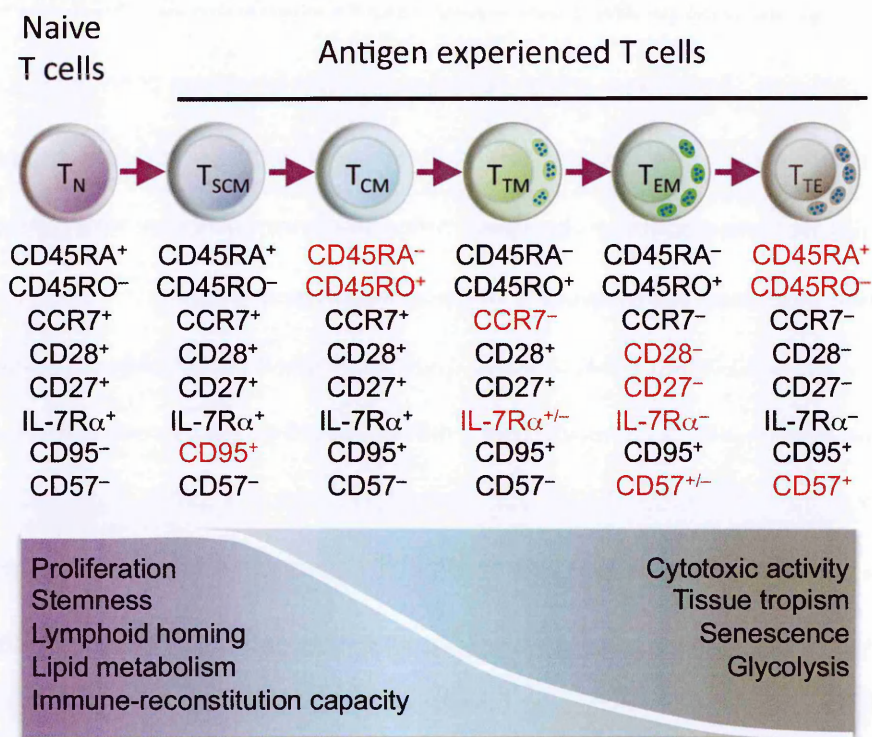


Figure 1: T cell differentiation process, functions and phenotypic features of T cell subsets. Polychromatic flow cytometry analysis allows the identification of each T subset in the T cell compartment. Each T subset is characterized by the expression of specific markers. In

red, the markers that change during differentiation from the previous subset. Going from less differentiated T cells to more differentiated ones, functional abilities also change. Modified from Mahnke et al., Eur J Immunol, 2013⁷⁵.

Each T cell subset can be distinguished phenotypically by the expression of surface molecules, as revealed by flow cytometry^{72,83} (Figure 1). These markers include molecules involved in T cell activation, such as the splicing variants of the tyrosine phosphatase CD45R, the long isoform (CD45RA) and shortest isoform (CD45RO). The former isoform is characteristic of less differentiated T cells (T_N and T_{SCM}), while the latter is expressed on more differentiated T cells (T_{CM} , T_{TM} , T_{EM}) with the exception of T_{TE} . T_{TE} , which are more frequent in the $CD8^+$ than in the $CD4^+$ T compartment, are able to re-express the naïve marker CD45RA, although they are distinguished from T_N by the absence of other naïve markers (e.g. CC chemokine receptor (CCR)-7, CD27, CD28 and $IL7R\alpha$), the expression of senescence markers (e.g. the killer cell lectin-like receptor -1 (KLRG-1)⁸⁴ and CD57, a marker of terminal differentiation⁸⁰) and the phosphorylation of histone H2AX^{72,85,86}. Among the markers expressed by memory T cells, CD95 is essential to distinguish T_{SCM} from T_N : it is expressed on T_{SCM} but not on T_N ⁷¹⁻⁷³.

Other markers involved in the co-stimulation and survival of T cells, which permit the discrimination of specific T subsets, include the TNF receptor superfamily member CD27 and CD28, the receptor for CD80 and CD86. The expression of the molecules is down-regulated in the late stages of differentiation (T_{EM} , T_{TE}). Homing receptors also have an important role in the identification of subsets with different capabilities to localize to peripheral tissue: CCR7 and the cell adhesion molecule L-selectin (CD62L), both necessary for homing to high endothelial venules in the lymph nodes, are expressed at high levels in T_N ^{66,67,72,74}. In contrast, memory cells are split into $CCR7^+CD62L^+$ (T_{SCM} and T_{CM}) and $CCR7^+CD62L^-$ (T_{TM} , T_{EM} and T_{TE}). In contrast to CCR7 and CD62L, the expression of

CCR5 and the chemokine (C-X-C motif) receptor 3 (CXCR3), which allow migration to inflamed tissues, are increased on more differentiated T cells (T_{CM} , T_{TM} , T_{EM}) but largely absent on T_N and T_{SCM} .

T cells subsets respond differently to cytokines due to the differential expression of cytokine receptors. Among them, CD127, the α -chain of the IL-7 receptor (IL-7R α), and CD122, the β chain of the IL-2/IL-15 receptor complex, have important roles in T cell development and homeostasis⁷². CD127 is prevalently expressed on less differentiated cells, which are more sensitive to IL-7. In contrast, more differentiated cells express CD122 and respond better to IL-15^{66,67,72,74}.

Despite the high number of markers differentially expressed by T cell subsets, a minimal set of 4 markers can be used to identify unequivocally T_N , T_{SCM} , T_{CM} , T_{TM} , T_{EM} and T_{TE} within the $CD4^+$ and in $CD8^+$ T cell compartments: CD45RO, CD95, CCR7 and CD27. Additional markers are redundant and do not provide additional benefits in the identification of such cells⁷².

The following section describes the functions and homeostatic properties of the different T cell subsets.

1.4.2 Homeostasis of human T cells

It is well known that the number of T lymphocytes in the body is tightly controlled by a mechanism called homeostatic proliferation⁸⁷, which ensures a slow turnover in the absence of cognate antigen stimulation⁸⁸. The maintenance and homeostatic proliferation of T cells is regulated by complex mechanisms which involve contacts with both self-pMHC and members of the common gamma chain (γ_c) cytokines, such as IL-2, IL-4 and, especially, IL-7 and IL-15^{61,89}.

The important role of TCR-self-pMHC interactions for the maintenance of homeostasis was observed about 10 years ago. The lifespan of naive $CD4^+$ and $CD8^+$ T cells was shortened when these cells were deprived of contact with self-

pMHC⁶², thus suggesting that self-pMHC complexes are crucial for maintaining the peripheral T cell pool. Indeed, in MHC class I or class II deficient mice, respectively CD8⁺ and CD4⁺ T_N were not able to survive⁹⁰⁻⁹⁶. However, the above-mentioned studies were performed in lymphopenic mouse models. Using non-lymphopenic model systems, other research groups found normal maintenance of CD4⁺ T cells^{61,87,91,97}. Therefore, the effect of homeostatic stimuli differs when the physiological environment changes. However, irrespective of lymphopenic or normal physiological conditions, it was observed that self-pMHC had an important role in T_N homeostasis and, in particular, T_N, compared to memory cells, are more dependent on self-pMHC to undergo homeostatic proliferation^{61,98}. The homeostatic proliferation of memory CD4⁺ and CD8⁺ cells is largely MHC-independent⁹⁹. Memory CD8⁺ and CD4⁺ T cells persist indefinitely in MHC I^{-/-} or MHC II^{-/-} mice, respectively, and retain effector functions upon antigen re-encounter.

Despite the controversial data about the role of self-pMHC interactions in homeostatic proliferation, researchers agree that cytokines have an important role in this regard. Both naïve and memory cells need common γ c cytokine signals to undergo homeostatic proliferation, despite differences between naïve and memory cells as regards reliance on these signals⁶¹. Naïve cells are more dependent on IL-7 compared to the other subsets to survive and proliferate⁶¹.

IL-7 is a cytokine produced by non-haematopoietic stromal and epithelial cells of the BM and thymus during lymphopoiesis as well as by non-haematopoietic and stromal cells of secondary lymphoid tissues, liver and intestine¹⁰⁰. T cells respond to IL-7 through the IL-7R. This receptor is composed of 2 subunits: the common γ c CD132, which is shared with the IL-2, IL-4, IL-9 and IL-15 receptors, and the α -chain (CD127), which confers cytokine specificity. CD127 is expressed by naïve and memory T cells, but is down-regulated in terminally-differentiated memory cells

(T_{EM} and T_{TE})¹⁰¹, as depicted in Figure 1. Its essential role was discovered several years ago by the finding that abrogation of contact with IL-7, either by adoptive transfer of T cells into IL-7 deficient hosts or by injection of an IL-7 blocking antibody into normal mice, reduced the survival of T_N⁸⁹. Additional experiments showed that overexpression of IL-7 in mice increased the size of the T_N pool¹⁰².

In lymphopenic conditions, the availability of IL-7 increases because of diminished usage by T cells. Increased concentrations of IL-7 induce a potent downstream signal that amplifies the TCR signalling resulting from contact with self-pMHC ligands¹⁰³. In this context, T_N proliferation is accompanied by the gradual differentiation in memory cells¹⁰³. IL-7-induced T_N proliferation is dampened by the presence of memory T cells, which compete for IL-7 signals¹⁰⁴.

In lymphopenic conditions, other cytokines have a role in the proliferation of T_N, in particular IL-15 and IL-2. These cytokines are structurally and functionally related and share two of their three receptor chains, the IL-2/15 R β (CD122) and the common γ chain (CD132).

IL-15 is produced primarily by DCs, monocytes and epithelial cells in the presence of inflammatory signals¹⁰⁵. It was initially characterized as a soluble molecule. Later it was shown that IL-15 also exists as a membrane-bound form, which is the prevalent form *in vivo*. In the membrane-bound form, IL-15 is complexed with high affinity to the α chain of its receptor (IL-15R α) and binds target cells according to a mechanism known as trans-presentation⁸¹. If non-complexed with IL-15R α , IL-15 is also able to bind to the 15R $\beta\gamma$ signalling complex with intermediate affinity¹⁰⁶.

Because IL-2 and IL-15 share receptor subunits (CD122 and CD132), both these cytokines have several shared downstream effects¹⁰⁷, including induction of the anti-apoptotic molecule Bcl-2, activation of the mitogen-activated protein kinase

(MAPK) pathway and phosphorylation of lymphocyte-activated protein tyrosine kinase (Lck) and spleen tyrosine kinase (Syk), which lead to cell proliferation and maturation¹⁰⁶. However, these cytokines are not functionally redundant¹⁰⁷. Mice deficient in IL-2 or IL-15 have different phenotypes, and administration of IL-2 and IL-15 to mice and primates leads to distinct immunological outcomes^{108,109}. Although both cytokines stimulate lymphocytes, IL-2 favours the homeostasis of regulatory T cells and regulates the differentiation of helper T cells¹¹⁰, whereas IL-15 favours the expansion of CD8⁺ memory cells¹⁰⁸.

The important role of IL-15 was demonstrated in studies where memory cells were transferred in IL-15 deficient mice. In these hosts, memory cells failed to undergo homeostatic proliferation and died rapidly¹¹¹. The increased expression of IL-15R α and IL-2/15R β -chain on more differentiated T cells (Figure 1) further indicates that IL-15 plays an important role in the homeostatic proliferation of memory cells rather than of naïve cells⁸¹.

To evaluate the role of cytokine stimulation on T cell subsets, Geginat and coworkers evaluated, *in vitro*, the acquisition of some phenotypic and functional features of purified T subsets isolated from human peripheral blood mononuclear cells (PBMCs). They compared cytokine and antigenic stimulation. The latter was provided by mature allogeneic DCs, whereas cytokine stimulation was provided mainly by IL-7, IL-15 and IL-2⁸¹. In the absence of antigen stimulation, the response to IL-7 is comparable and low in all subsets. In contrast, the response to IL-15 increases with progressive differentiation and the response to cytokine stimulation is boosted when IL-7 is added to the culture. Moreover, IL-2 selectively stimulates memory T cells and boosts responses to IL-7 and IL-15⁸¹. In regard to antigenic stimulation, T_{CM} and T_{EM} show a higher sensitivity compared to T_N and are less

dependent on co-stimulation. In particular, T_{CM} produce mainly IL-2^{67,112}, whereas T_{EM} mainly produce IFN- γ and are endowed with cytolytic capacity^{81,113}.

Observing the cell phenotype after antigen and cytokine stimulation, T_N , T_{CM} , T_{EM} and T_{TE} give rise to a rather homogeneous population of CCR7⁻ CD45RA⁻ effector T cells. In contrast, after cytokine stimulation, all subsets are able to generate more differentiated cells in different combinations, while a fraction maintains the original phenotype, thus indicating the capacity to self-renew. These results suggest that both antigenic and cytokine stimulation induce the proliferation and differentiation of each subset to more differentiated T cells⁸¹. Among T cell subsets, T_{SCM} self-renew and generate more differentiated progeny at a higher efficiency compared to T_{CM} in response to TCR stimulation (via α -CD3/CD2/CD28 exposure) and homeostatic signals (via IL-15 stimulation)⁷¹. Following IL-15 stimulation, about 60% of T_{SCM} maintain their phenotypic markers compared to 30% of T_{CM} . After α -CD3/CD2/CD28 exposure, a fraction of T_{SCM} gradually up-regulates the memory marker CD45RO while down-regulating CD62L and CCR7 over several divisions, giving rise to T_{CM} and T_{EM} . Conversely, T_{CM} are able to maintain their original phenotype only in a smaller fraction compared to T_{SCM} and could only generate T_{EM} but not T_{SCM} , thus indicating that, at least *in vitro*, “de-differentiation” does not occur⁷¹.

1.4.3 T cell recovery following immune ablation

As reported in previous sections, transient T cell immunodeficiency is the inevitable consequence of high-dose chemotherapy for blood and solid cancers. Following immune ablation, the body attempts to restore the T cell compartment but the recovery of T cell populations is delayed compared to that of myeloid, NK or B cells¹¹⁴.

Several studies have reported that T cell function is often compromised, even after normal lymphocyte numbers have recovered¹¹⁵⁻¹¹⁷, thus leading to serious clinical consequences, including a limited response to vaccines, reduced resistance to infection, tumor relapse and the development of autoimmunity¹¹⁶⁻¹¹⁹. T cell immune reconstitution is generally dependent on the contribution of two primary pathways: the generation of new T cells from progenitors via thymopoiesis (“thymic-dependent”)¹²⁰⁻¹²² and the peripheral expansion of residual mature lymphocytes by antigenic, allogeneic or cytokine-driven stimulation^{121,122}.

The peripheral expansion pathway characterizes the early phase of immune reconstitution (up to 1 year post-transplantation): the T cells that survived chemotherapy or were adoptively transferred via BMT undergo vigorous expansion in response to increased homeostatic signals, namely those induced by IL-7 and IL-15, or stimulation by exogenous antigens^{121,123}. The “thymic-dependent” phase occurs several months post-BMT and is characterized by the slow recovery of T cell lymphopoiesis leading to the production of new T_N¹²⁴.

The relative contribution of each pathway depends on host age (indeed, thymic activity decreases with age), residual T cells, homeostatic cytokine levels and endogenous antigenic stimulation¹²². Each pathway contributes to generate a different TCR repertoire of T cells: a more diverse repertoire through thymopoiesis and homeostatic expansion and an oligoclonal repertoire through antigen stimulation^{121,122}. As indicated above, the CD4⁺ and CD8⁺ T cell compartments are highly heterogeneous and comprise subsets that respond differently to the mechanisms driving the reconstitution process. A clear understanding of the mechanisms that govern immune reconstitution is therefore important to predict T cell functionality after lymphopenia. However, a comprehensive analysis of T cell

subset immune reconstitution has never been reported, especially in the setting of haploidentical donors and reduced conditioning regimen.

1.5 Adaptive immunity: B cell compartment

Like T cells, B cells belong to the adaptive arm of the immune system. They are characterized by immunoglobulin (Ig) receptors that are involved in the recognition of intact antigen in its native form¹²⁵. Igs are generated by somatic recombination, whereby germline genes are re-arranged to create diverse receptor sequences.

1.5.1 Homeostasis and maturation of human B cells

Human B lymphocytes differentiate in the BM from common lymphoid progenitors.¹²⁶ Pre-B cells arise from progenitor (pro-B) cells that express neither the pre-B-cell receptor (pre-BCR) nor surface Ig. Early B cell development is characterized by the functional rearrangement of the Ig loci which culminates in the development of a diverse repertoire of functional Ig heavy and Ig light chain loci via V(D)J rearrangement¹²⁷. The generated heavy and light chains constitute the B-cell receptor (BCR), whose expression is a requisite for B-cell development and allows survival and re-circulation in the periphery^{128 127}.

The recent BM emigrants, called transitional B lymphocytes, are characterized by the expression of CD38, CD10, CD24 and IgD^{129,130}. These immature B cells exit the BM and mature stepwise in the periphery, a process that can be distinguished on the basis of multiple phenotypic markers: CD5^{129,131}, CD21¹³², CD24, CD38 or CD10^{133,134}. While progressing from the most immature CD21⁻CD5⁺CD38^{bright}CD24^{bright}CD10⁺ transitional T1 cells to CD21⁺CD5⁺CD38^{dim}CD24^{dim}CD10⁺ T2 and CD21⁺CD5⁻CD10⁻ naïve cells^{129,130}, B

cells acquire proliferative and survival capacity and the potential to produce antibodies as well as undergo Ig class switching¹³².

Mature naïve B cells migrate to secondary lymphoid structures. Once B cells encounter cognate antigen, often through interactions with CD4⁺ T cells or DCs, they become activated, undergo clonal expansion¹³⁵ and release IgM. During clonal expansion, B cells may undergo isotype switching such that they express IgG, IgA or IgE on their cell surface. Moreover, somatic hypermutation, which consists of DNA mutations in the variable regions, may occur to increase the avidity of the antibody/epitope interaction³⁷. These cells are the antigen-experienced B cells, which can leave the lymph node and migrate to the PB or lymphoid tissues¹³⁶. B cell proliferation upon antigen encounter and T cell help is a key step in the humoral immune response^{137,138}.

In addition to the classical B cell maturation process, another T-independent mechanism has been extensively described. Indeed, transitional B cells may also mature as marginal zone (MZ) B cells, which have mixed attributes of naïve and memory B cells¹³⁵. Transitional B cells and MZ B cells respond to T cell-independent antigens¹³⁹ and, together with mature naïve B cells, constitute the pre-immune/natural effector compartment.

1.5.2 B cell reconstitution following transplantation

Following BMT and HSCT, levels of circulating B cells are generally undetectable within the first 2-3 months post-transplantation. Subsequently, B cells expand and circulating B cell counts transiently become supranormal by 1–2 years after grafting, finally stabilizing years after the transplantation procedure¹⁴⁰⁻¹⁴³. In most published reports, all circulating B lymphocytes are of donor origin after T cell-replete transplant with high intensity conditioning regimen; conversely, after T cell-

depleted transplantation and after transplantation with low intensity or no conditioning regimen, a variable degree of incomplete chimerism of B cells is frequently established^{120,143}. Generally, the rise is faster in young versus old individuals¹⁴⁴, in autologous versus allogeneic marrow recipients and in patients who do not develop GVHD^{140,145}. Indeed, GVHD and/or its treatment may hamper B-lymphopoiesis^{143,146,147}. B cell recovery is also similar between myeloblastic and non-myeloablative regimens, indicating no beneficial effects of reduced chemotherapy dose in this regard¹⁴⁸.

Independently of the transplantation procedure, many studies reported that B cell development after transplantation recapitulates the normal ontogeny in terms of circulating cell types and numbers. Immunophenotypic analysis of B cell recovery in the early 1990's reported the presence of undifferentiated B lymphocytes in the early phases post-transplant, as revealed by the expression of immature markers on the cell surface^{140,142}. Post-transplant, donor B cells emerge initially with a naïve phenotype, lacking somatically mutated V(D)J genes¹⁴⁹⁻¹⁵¹ and produce IgM and IgD rather than IgG or IgA^{140,152,153}.

Memory B cells are scarce in the first 1–2 years post-transplant and develop up to 5 years later^{150,154}. Consistent with this, it is reported that IgM levels recover first, within 2-6 months^{43,155}, followed by IgG, which approaches normal levels between 3 and 18 months following transplantation, and IgA, the reconstitution of which may be delayed for up to 3 years^{43,155}.

Ig levels are primarily of recipient origin early post-transplant, even in patients with complete chimerism of lymphocytes. This is because host plasma cells are relatively resistant to current preparative regimens and may persist for up to 2 years after transplant^{143,156,157}. However, the delay in conversion to only donor-type antibodies is also a consequence of the compromised function of B cells due to the

limited repertoire of T cells available to recognize and signal to their B cell counterparts³⁷.

Memory B cells show skewing of Ig heavy chain complementary-determining region 3 (CDR3) loop patterns^{158,159} and a decreased rate of somatic hypermutation in mature B cells¹⁵¹. Thus, following transplantation, B cell immunity is impaired due to prolonged low levels of circulating B cell numbers, a relative deficit of mature B cells due to decreased isotype switching and a diminished ability to undergo somatic hypermutation.

The prolonged presence of immature and naïve B cells associated with the low frequency of fully functional memory B cells has been proposed to be, at least in part, responsible for the hyporesponsiveness of transplanted patients to vaccinations, even after normal B cell numbers have been achieved^{120,160-162}.

2 Aim of the study

HaploBMT combined with pt-Cy is changing the perspective of allogeneic transplantation because it allows the identification of suitable donors for patients who lack of HLA-matched donor. The infusion of unmanipulated BM, as it occurs in this protocol, would potentially allow the transfer of mature immune cells to the patient. Multiple subsets of T and B cells with different immune functions are infused with the BM, but it is unclear at present to what extent these cells can persist in the lymphopenic environment. Moreover, it remains to be determined whether pt-Cy spares non-alloreactive cells and thus favours immune reconstitution.

The main aim of this study is to evaluate the mechanisms that underlie reconstitution of the adaptive immune system following haploBMT combined with pt-Cy. Specifically, we used 18-color polychromatic flow cytometry and cell sorting, as well as molecular technologies (polyclonal and antigen-specific level,) to determine the persistence and expansion of T and B cells adoptively transferred with the graft.

We concentrated our efforts during the first few days/weeks post-BMT to investigate the mechanism of action of pt-Cy. Longitudinal analyses of blood samples were exploited to determine lymphocyte dynamics over time and to follow the maturation process of recovering lymphocyte populations.

In the case of T cells, we unconventionally exploited infection matched and mismatched donor/recipient pairs to investigate the contribution of cognate antigen to the persistence and expansion of transferred pathogen specific T cells.

3 Methods

3.1 Experimental design

A total of 28 consecutive haploBMT patients were studied and none of them were excluded *a priori* from the analysis. All experiments display biological replicates from different patients or healthy donors that were randomly selected, unless specified (such as for the study of antigen-specific responses). The number of replicates used in experiments is indicated in the figure legends. PB specimens from patients were collected longitudinally, frozen then stored in liquid nitrogen and analyzed in batches by flow cytometry to minimize variability. Buffy coats from healthy donors were processed independently and used in control experiments.

3.2 Study subjects and transplantation procedures

Twenty-eight patients were treated in the Haematology and Bone Marrow Transplant Unit of Humanitas Cancer Center according to the haploBMT protocol established by Luznik *et al.*³. Briefly, the conditioning regimen consisted of 14.5 mg/kg Cy on day (d) -6 and d-5, 30 mg/m² fludarabine from d-6 to d-2 and low-dose total body irradiation (200 CentiGray) on d-1. The GVHD prophylaxis consisted of 50 mg/kg Cy administered on d3 and d4, tacrolimus (FK 506) and mycophenolate mofetil (MMF). FK (total dose 1 mg) was administered as a continuous infusion. MMF was administered orally at 15 mg/kg three times per day until d35 and then stopped. FK and MMF were started on d5. FK was tapered by d180. Granulocyte-colony stimulating factor (G-CSF) was started on d5 in all patients. HLA typing was performed on selected BM donors, who were considered haploidentical when at least one allele from the *HLA-A*, *HLA-B*, *HLA-Cw*, *HLA-DRB1* and *HLA-DQB1* loci matched the recipient. Additional details of the clinical protocol are available at

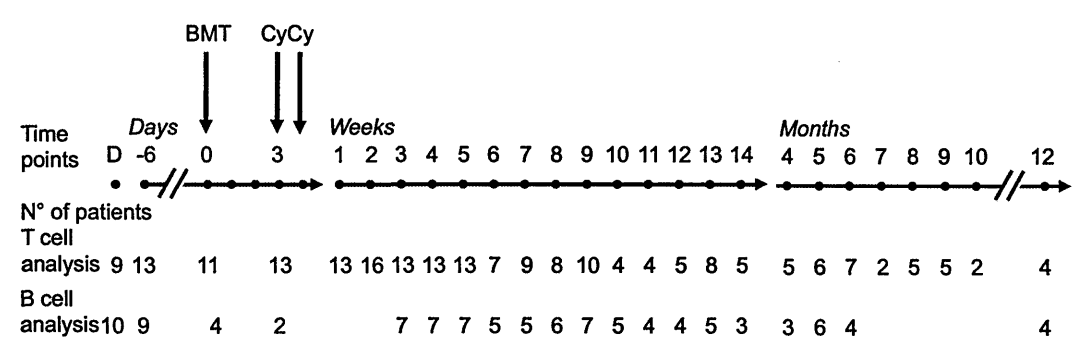
www.clinicaltrials.gov, protocol numbers NCT02049424 and NCT02049580. Patient characteristics are listed in Table 1. Patients and donors signed consent forms in accordance with the Declaration of Helsinki and with a related clinical and experimental protocol approved by the Institutional Review Board of Humanitas Research Hospital (Prot. Nr CE Humanitas 222/14).

No.	Disease	Donor	Sex D/R	Age D/R	CMV D/R	Disease status before transplant	Indication for transplant	aGVHD (grade, localization)	agVHD (therapy)	cGVHD (grade, localization)	cGVHD (therapy)	Infections/Virus reactivations	B cell Follow-up (weeks)	T cell Follow-up (weeks)	Disease status	Reason for stopping follow-up
1	HL	Father	M/M	52/27	+/+	PR	RAA	NA		NA		H1N1	-	5	NA	Deceased
2	HL	Son	M/F	24/46	+/+	CR	TAA	-		-		Non albicans candida	-	5	CR	Graft failure
3	HL	Mother	F/F	51/19	+/+	CR	RAA					EBV	26	53	CR	End of the study
4	HL	Father	M/F	52/22	-/+	CR	RAA	2, Skin	Methylprednisolone	Mild, GI tract	Budesonide	Parainfluenza virus, CMV, EBV	26	55	CR	End of the study
5	NHL	Sister	F/M	64/53	+/+	PR	RAA	-		-		JC	9	9	NA	Deceased
6	HL	Brother	M/M	47/45	-/+	CR	RAA	2, Skin	ECP	Mild, Skin	Prednisone	S. epidermidis, BK, EBV	22	36	CR	End of the study
7	NHL	Sister	F/F	49/49	+/+	PR	RAA	AS		-		CMV	-	6	PR	Consent withdrawal
8	HL	Mother	F/F	61/33	+/+	CR	RAA			-		-	25	25	CR	Other*
9	NHL	Brother	M/M	47/45	+/+	CR	HR	AS		NA		ESBL E. coli	5	5	NA	Consent withdrawal
10	NHL	Brother	M/M	62/57	+/-	CR	RAA	2, Skin	Methylprednisolone	-		S. aureus, S.epidermidis	-	13	CR	Consent withdrawal
11	HL	Brother	M/M	29/34	-/-	CR	TAA	2, Skin	ECP	-		-	22	50	PD	Deceased
12	NHL	Son	M/F	23/44	+/+	CR	TAA	NA		NA		E. faecalis, A. fumigatus	-	6	NA	Deceased
13	NHL	Brother	M/M	46/54	+/+	PR	TAA	1, Skin	Tacrolimus	-		ESBL E. coli, CMV	26	27	CR	Other*
14	HL	Son	M/F	24/57	-/+	CR	TAA	-		-		ESBL E. coli, HHV6	-	6	CR	Graft failure
15	NHL	Brother	M/F	62/57	+/+	CR	RAA	AS		-		C. difficile, CMV, E. coli	9	9	CR	Consent withdrawal
16	HL	Mother	F/F	60/24	+/+	CR	TAA	NA		NA		RSV	-	4	NA	Deceased
17	HL	Mother	F/M	45/24	+/+	PD	RAA	-		-		CMV	8	8	PD	Consent withdrawal
18	NHL	Father	M/M	51/25	+/+	CR	TAA	1, Skin	-	-		-	-	53	CR	End of the study
19	HL	Sister	F/F	38/47	+/+	CR	RAA	1, Skin	-	-		-	-	36	CR	Other*
20	NHL	Brother	M/M	50/51	-/+	PR	Ref	-		-		ESBL E. coli, CMV, BK	-	23	SD	Other*
21	HL	Sister	F/F	32/28	+/-	CR	RAA	-		-		-	-	3	CR	Other*
22	HL	Sister	F/M	21/25	+/-	CR	RAA	AS		AS		-	-	4	CR	Consent withdrawal
23	HL	Mother	F/F	54/26	+/+	PR	Ref	NA		NA		-	-	3	NA	Deceased
25	NHL	Son	M/M	35/62	+/-	PR	RAA	2, Skin	ECP	Mild, Skin	-	S. maltophilia, CMV	-	37	CR	End of the study
26	NHL	Sister	F/F	49/42	+/+	PR	RAA	1, Skin	-	-		-	-	33	CR	End of the study
27	HL	Cousin	M/F	48/40	+/+	PR	RAA			-		CMV, EBV	-	25	CR	Other*
28	HL	Father	M/F	51/21	-/-	PR	TAA	1, Skin	-	NA		-	-	6	PD	Consent withdrawal
29	HL	Father	M/M	51/24	+/+	SD	RAallo	-		-		-	-	4	NA	Deceased

Table 1: Characteristics of the patients enrolled in the study. Abbreviations: HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; D/R, donor/recipient; CMV, Cytomegalovirus; PR, partial remission; CR, complete remission; PD, progressive disease; SD, stable disease; RAA, Relapse after autologous; TAA, Tandem autologous-allogeneic; HR, High-risk; Ref, Refractory; RAallo, relapse after allogeneic transplantation; NA, not applicable; AS, developed after sampling was stopped; ECP, Extracorporeal photochemotherapy; NA, not applicable; GI, Gastrointestinal; EBV, Epstein-Barr virus; JC, John Cunningham polyomavirus; S. Epidermidis, Staphylococcus Epidermidis; BK, BK polyomavirus; ESBL E. Coli, Extended-spectrum beta-lactamase-producing Escherichia Coli; S. aureus, Staphylococcus aureus; E. faecalis, Enterococcus faecalis; A. Fumigatus, Aspergillus Fumigatus; HHV6, Human Herpes Virus 6; C. Difficile, Clostridium Difficile; RSV, Respiratory Syncytial Virus S. Maltophilia, Stenotrophomonas Maltophilia; HSV, Herpes Simplex Virus; P. aeruginosa, Pseudomonas aeruginosa. **Notes:** *: the patient stopped coming to the clinic.

3.3 Sample collection

Peripheral blood (20 to 100 mL) and BM (5 mL) specimens were obtained from the Haematology and Bone Marrow Transplant Unit of Humanitas Cancer Center. PB was drawn in heparinized tubes from donors and recipients before transplantation and from recipients every week until d98, then every month until one year after transplantation (Figure 2). BM was also collected during the aspiration procedure. Buffy coats of healthy donors were obtained from S. Gerardo Hospital (Milan, Italy) under Institutional Review Board exemption. PBMCs and BM cells were obtained by Ficoll-Paque Premium (GE Healthcare) density gradient centrifugation and frozen in liquid nitrogen according to standard procedures¹⁶³.



3.4 Polychromatic flow cytometry, phenotyping and cell sorting

For analysis of the T cell compartment, the following anti-human monoclonal antibodies (mAbs) were used: CD3 (clone SP-34.2 and OKT3), CD4 (SK3 and OKT4), CD8 (RPA-T8), CD45RA (HI100), CD45RO (UCHL1), CCR7 (150503), CD27 (O323 and 1A4CD27), CD95 (DX2), CD31 (WM59), Ki-67 (B56), CD57 (HNK-1), HLA-DR (G46-6), IL-2 (MQ1-17H12), IFN γ (B27), TNF (Mab11), CD127 (A019D5), CD25 (M-A251), HLA-A2 (BB7.2), HLA-B7 (BB7.1, from Millipore) and CD14 (M5E2). For analysis of the B cell compartment, the following anti-human mAbs were used: CD19 (HIB19), CD20 (2H7), CD10 (HI10a), CD38 (HIT2), CD21 (B-ly4), IgM (G20-127) IgD (Polyclonal and IA6-2), IgG (G18-145), CD5 (UCHT2), CD27 (1A4LDG), CD14 (TüK4) Life Technologies, CD3 (OKT3), CD8 (RPA-T8), CD4 (M-T477), Ki-67 (B56), HLA A2 (BB7.2).

Fluorochrome-conjugated mAbs were purchased from BD Biosciences, Biolegend, BD Horizon, BD Pharmingen, Dako and Life Technologies, or conjugated in the laboratory of Dr. Mario Roederer from purified unlabeled mAbs (BD Biosciences). In-house conjugation was performed according to the protocols reported at <http://www.drmmr.com/abcon>.

All antibodies were titrated on human PBMCs and used at the concentration giving the highest signal-to-noise ratio over background, as described¹⁶⁴. Co-staining with multiple reagents at the same time validated the specificity of the staining. Anti-HLA class I antibodies were tested on individuals with or without expression of the relevant HLA molecule, as determined by HLA typing.

Frozen cells were thawed in RPMI1640 supplemented with 10% fetal calf serum, 1% penicillin-streptomycin and 1% L-glutamine (all from Life Technologies) containing 50 U/mL Benzonase nuclease (EMD Biochemicals) and stained.

In all experiments, cells were stained for 15 min at room temperature (RT) with a live/dead fixable dead cell stain kit (Life Technologies) to eliminate dead cells, which may influence the analysis. After that, cells were stained with for 20 min at RT with a combination of mAbs specific for surface proteins, as described previously⁷². Chemokine receptor expression was revealed by incubating cells at 37°C for 20 min with the relevant mAb. The Cytofix/Cytoperm kit (BD Biosciences) was used according to the manufacturer's protocol to detect intracellular Ki-67 expression¹⁶⁵.

T cell subsets were defined as follows, unless indicated otherwise: recent thymic emigrant T (T_{RTE}) $CD4^+$ cells, $CD45RO^-CCR7^+CD27^+CD45RA^+CD95^-CD31^+$; T_N $CD4^+$ cells, $CD45RO^-CCR7^+CD27^+CD45RA^+CD95^-CD31^-$ (for $CD8^+$ T cells, T_N were defined as $CD45RO^-CCR7^+CD27^+CD45RA^+CD95^-$, irrespective of CD31 expression); T_{SCM} , $CD45RO^-CCR7^+CD27^+CD45RA^+CD95^+$; T_{CM} , $CD45RO^+CCR7^+$; T_{TM} , $CD45RO^+CCR7^-CD27^+$; T_{EM} , $CD45RO^+CCR7^-CD27^-$; and, T_{TE} , $CD45RO^-CCR7^-$. Figure 3 displays the gating strategy used for analyzing T cell subsets.

B cells were defined as follows: transitional B cells, $CD38^{bright}CD10^+$ cells; mature B cells, $CD38^{dim}CD10^-$. In the mature B cell compartment, naïve B cells were defined as IgM^+ or IgD^+ , and memory B cells were defined as IgM^-IgD^- and include IgG^+ and IgG^- cells. In the transitional compartment, the following subsets were also defined: T1 ($CD5^+CD21^-$), T2 ($CD5^+CD21^+$), T3 ($CD5^-CD21^+$) and $CD5^-CD21^-$ cells, here named T0. Figure 4 displays the gating strategy used for B cell subsets analysis.

To minimize technical variability, samples from the same patient were analyzed in batches. Samples were acquired on a LSRII or Fortessa flow cytometer (BD Biosciences), equipped for the detection of 20 parameters. A FACS Aria II cell

sorter (BD Biosciences) was used to separate cell subpopulations. Absolute counts of T and B cell subsets were obtained by calculating the percentage of live CD3⁺ and CD20⁺ cells respectively, within the lymphocyte gate. Absolute lymphocyte numbers per μ L of PB were obtained from the Flow Cytometry Core Facility of the Haematology and Bone Marrow Transplant Unit at the Humanitas Cancer Center.

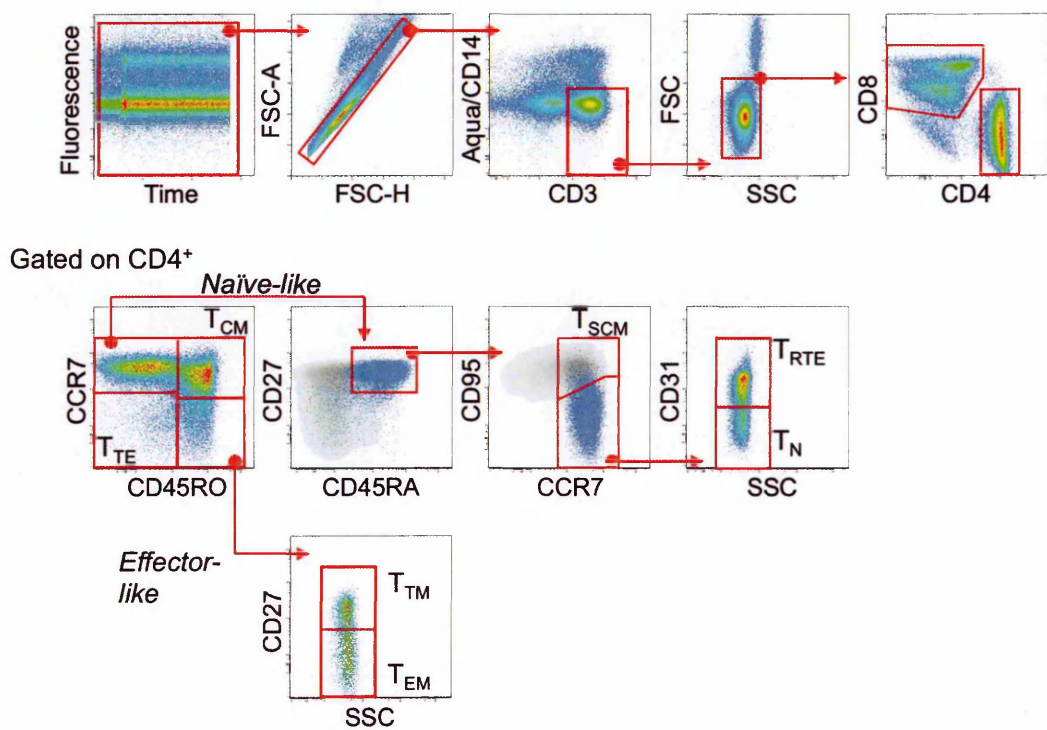


Figure 3: Gating strategy used to identify T cells at different stages of maturation by polychromatic flow cytometry. CD4⁺ and CD8⁺ T cells are identified by gating on CD3⁺, CD14⁻ and Aqua Live/Dead⁻ cells. Gating on CD4⁺ T cells, T_{TE} are identified as CD45RO⁻CCR7⁻; T_{CM} are CD45RO⁺CCR7⁺; effector-like cells are CD45RO⁺CCR7⁻ and further distinguished on the basis of CD27 expression as T_{TM} (CD27⁺) and T_{EM} (CD27⁻); T_{SCM} are CD45RO⁻CCR7⁺CD27⁺CD45RA⁺CD95⁺; T_{RTE} are CD45RO⁻CCR7⁺CD27⁺CD45RA⁺CD95⁻CD31⁺; T_N are CD45RO⁻CCR7⁺CD27⁺CD45RA⁺CD95⁻CD31⁻. For CD8⁺ T cells, T_N were defined as CD45RO⁻CCR7⁺CD27⁺CD45RA⁺CD95⁻, irrespective of CD31 expression. A similar gating strategy was used for CD8⁺ T cells.

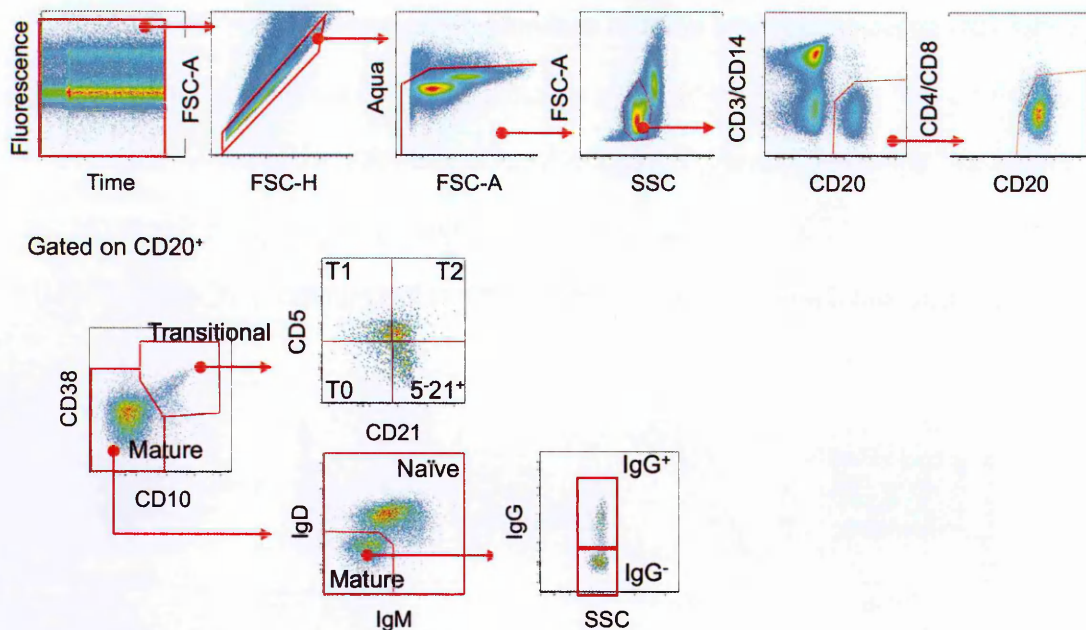


Figure 4: **Gating strategy used to identify B cells at different stages of maturation by polychromatic flow cytometry.** B cells are identified by gating on $CD20^+CD3^-CD14^-$ Aqua Live/Dead⁻ cells. Gating on $CD10^+CD38^+$ B cells, transitional B cells are identified. In this compartment, T1 ($CD5^+CD21^-$), T2 ($CD5^+CD21^+$), $CD5^-CD21^+$ and $CD5^-CD21^-$ cells, named T0, are distinguished. Mature B cells are identified as $CD38^{dim}CD10^-$. Gating on mature B cells, naïve B cells are defined as IgM^+ and/or IgD^+ , whereas memory B cells are defined as IgM^-IgD^- and include IgG^+ and IgG^- cells.

3.5 Analysis of donor chimerism

DNA was obtained from sorted live $CD3^+CD56^-CD20^-CD14^-$ T cells and sorted live $CD3^-CD56^-CD14^-CD19^+$ B cells from PB using a Qiaamp Blood Mini Kit (Qiagen) according to the manufacturer's instructions. T cells and B cells were sorted from three patients at the following time points post-haploBMT: d63, d91 and d126. DNA quantification was performed using a photometer (BioPhotometer, Eppendorf). Sixteen polymorphic short tandem repeat markers were amplified using a multiplex PCR fluorescent approach (AmpFISTR Identifiler Plus, Applied Biosystems) on an ABI 9700 thermal cycler (Applied Biosystems). The reaction mixture was set up using 25 ng DNA to evaluate at least 3,000 cells as described previously¹⁶⁶. At least five of these informative loci were amplified using a singleplex Quantitative Fluorescent PCR approach. Primer sequences were

obtained from the UniSTS database (www.ncbi.nlm.nih.gov). PCR products were run on a Capillary Electrophoresis System (ABI 310, Applied Biosystems) and analyzed using GENESCAN 3.1.2 software. Single PCR products were visualized in electropherograms as peaks variable in length and color. Residual mixed chimerism was calculated on the basis of the relative length of donor and recipient alleles as described previously¹⁶⁶.

3.6 T cell stimulations and treatments

PBMCs or sorted T cell subsets were seeded at a final density of 0.25×10^6 cells/mL unless indicated. Cell proliferation was determined by the analysis of 5- (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies) dilution. Cells were stained at 37°C for 7 min at a final concentration of 5 mM, washed extensively in complete medium and subsequently stimulated with 1 or 50 ng/mL rhIL-15 (Peprotech) for 8-10 days. Patients' PBMCs at different times post-haploBMT (n=5; d41, d53, d56, d57 and d65) were used to generate Figure 12B.

To induce effector cytokine production, PBMCs were cultured in 96-well plates (final volume: 200 μ L) and stimulated with phorbol 12-myristate 13-acetate (PMA; 10 ng/mL) and ionomycin (500 ng/mL, both from Sigma Aldrich) for 4 hr in the presence of GolgiPlug (BD Biosciences) according to the manufacturer's instructions. To detect antigen-specific T cells by intracellular cytokine staining, PBMCs were stimulated for 16 hrs with a CMV peptide pool spanning the entire region of the pp65 protein (15mer peptides overlapping by 11 residues; JPT Technologies; final concentration 1 mg/mL/peptide) or with VAXIGRIP® seasonal influenza vaccine (Sanofi; obtained from the local pharmacy; used at 1:40 vol/vol). Intracellular cytokine production was revealed as described previously¹⁶⁵.

In mixed lymphocyte reaction (MLR) cultures, highly-purified T_N and memory T cell subsets were cultured at a ratio of 1:1 with autologous APCs (auto-APCs) or MHC-mismatched APCs (allo-APCs) for the times indicated in the text. APCs were sorted from PBMCs as CD3⁺CD56⁺ mononuclear cells. To block APC:T cell interactions, anti-MHC class I and class II blocking antibodies were used (clones G46-2.6 and Tu39, respectively; both used at 10 mg/mL). Non-alloreactive T cells were identified by either gating on CFSE^{high} cells or on cells negative for both CD25 and CD69 activation markers.

3.7 Enumeration of antigen-specific T cells by MHC class I tetramers

The following recombinant pMHCI tetramers, produced as described previously¹⁶⁷, were used: HLA-A*0201 presenting CMV pp65₄₉₅₋₅₀₃ NLVPMVATV (NV9), HLA-A*0201 presenting influenza matrix protein₅₈₋₆₆ GILGFVFTL (GL9), HLA-A*0201 presenting melanoma antigen recognized by T cells 1 (MART-1)₂₆₋₃₅ ELAGIGILTV, HLA-A*0201 presenting Wilms' Tumor 1 (WT1)₃₇₋₄₅ VLDFAPPGA, HLA-A*0201 presenting WT1₁₂₆₋₁₃₄ RMFPNAPYL, HLA-A*0201 presenting WT1₂₃₅₋₂₄₃ CMTWNQMNL, HLA-A*0301 presenting influenza nucleoprotein₂₆₅₋₂₇₃ ILRGSVAHK (IK9) and HLA-A*B07 presenting CMV pp65₄₁₇₋₄₂₆ TPRVTGGGAM (TM10). The human CD1d tetramer loaded with the α-galactosylceramide analog PBS57 was provided by the NIH Tetramer Core Facility. Tetramers were conjugated with streptavidin-BV421 (Biolegend), PE (Sigma) and APC (Life Technologies). Tetramer staining was conducted at 37°C for 15 min. To detect WT1⁺ cells, tetramers recognizing 3 different WT1 epitopes were combined in the same channel. Given the large number of cells needed for the quantification of WT1⁺ T cells, PBMCs from blood bank donors instead of marrow donors were used.

CD8⁺ T cells were enriched by negative magnetic sorting (Stem Cell Technologies) from 10-30x10⁶ PBMCs prior to staining. Between 0.3 and 3x10⁶ total CD8⁺ T cells were acquired. For MART-1 and WT1, the percent threshold of positivity (0.003926 and 0.003705, respectively) was calculated by analyzing the 75th percentile of distributions resulting from the percentage of tetramer-binding cells in the CD4⁺ T cell compartment. Only one representative threshold is depicted in Figure 16D for simplicity.

3.8 Clonotypic analysis of antigen-specific CD4⁺ and CD8⁺ T cells

Preliminary peptide mapping experiments were conducted to identify donor and recipient CMV-specific CD4⁺ and CD8⁺ T cells responding to the same pool of 12 overlapping peptides spanning the CMV pp65 protein, as described¹⁶⁸. Aqua⁻CD14⁻CD3⁺CD4/CD8⁺ T cells producing IFN γ or TNF in the memory pool were subsequently sorted into 1.5 mL microfuge tubes (Sarstedt) containing 200 μ L of PBS. A median of 3901 (range: 712-30,030) specific cells were sorted per sample from up to 80x10⁶ PBMCs. Clonotypic composition was determined in collaboration with Professor David Price using a DNA-based multiplex PCR for *TCRB* gene rearrangements as described previously¹⁶⁹.

3.9 Statistical analysis

Analysis was performed using GraphPad PRISM (6.0b) and SPICE 5.22 software¹⁷⁰. The non-parametric paired Wilcoxon rank test and the unpaired Mann-Whitney test were used to compare two groups. Differences in the pie chart

distributions were calculated with SPICE software using a permutation test. P values are two-sided and were considered significant when ≤ 0.05 .

4 Results

4.1 Immune reconstitution is donor-dependent

In Luznik's protocol, a non–myeloablative conditioning regimen is used as chemotherapeutic treatment before transplantation. So, after BMT, donor immune cells infused with the transplant, as well as chemotherapy-resistant recipient immune cells, may coexist in the recipient. We sought to determine whether the reconstituting PB B cells in the transplanted patients derived from the donor or the recipient.

In collaboration with Dr. B. Cassani and Dr. G. Bulfamante (Unit of Pathology of San Paolo Hospital, Milan, Italy), we performed donor-recipient chimerism analysis of PB leukocytes of all patients who had received haploBMT, within 120 days post-transplantation. This analysis revealed that more than 98% of the circulating leukocytes in the recipient carried donor alleles, as also reported by our clinical team²⁶.

However, to exclude a bias due to the presence of high proportions of non-T cells and non-B cells that could misread the actual T and B cell chimerism, we sorted live $CD3^+CD56^-CD20^-CD14^-$, either expressing CD4 and CD8, and live $CD3^-CD56^-CD14^-CD19^+$ circulating B lymphocytes to high purity from 3 patients during immune cell reconstitution (d63, d91 and d126) and performed chimerism analysis on the sorted cells (Figure 5). The data obtained were very similar to those obtained with total PBMCs and confirmed that more than 99% of the circulating T and B cells detected in the recipients derived from the marrow donors (Figure 5). Finally, we also measured the donor chimerism at the single cell-level by the flow cytometry using antibodies specific for the mismatched HLA (e.g. HLA-A*02, HLA-B*07) combined with antibodies to identify T cells and B cells, thus validating the

donor origin of the reconstituting PB T and B cells following haploBMT. These data are reported in the following sections (Figure 6B, Figure 17B). Overall the results indicate that only donor T and B immune cells play a role in the peripheral immune recovery.

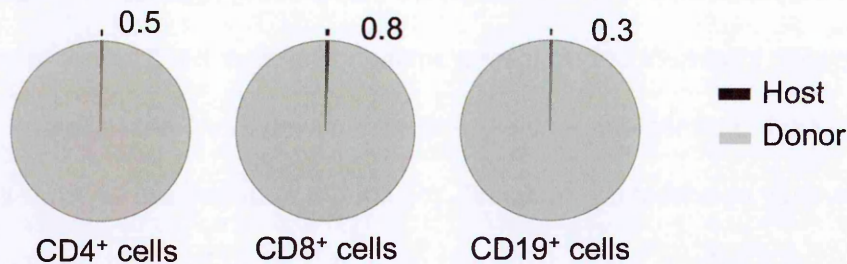


Figure 5: Proportions of donor-recipient T and B cells 2 months after transplant. Proportions of donor-derived (gray) and residual host (black) cells in FACS-sorted CD4⁺ and CD8⁺ T cells and in FACS-sorted CD19⁺ B lymphocytes. The reported data are the median of 3 BM recipients. Cells were sorted from the 3 patients at d62, 91 and 126 after BMT, respectively. Numbers indicate the proportion of residual recipient cells.

4.2 T cell reconstitution after transplantation

4.2.1 High frequency of T_{SCM} in the PB before reconstitution

It is well known that in response to antigen or lymphopenia, T cells undergo activation and rapidly start to proliferate and acquire an effector phenotype, represented by the lack of CD45RA and CCR7 and by the expression of CD45RO^{75,114,121,171}. At d3 following BM infusion, the vast majority of circulating T cells expresses markers of proliferation (Ki-67) and activation (HLA-DR⁺; Figure 6A). These cells acquire the CD45RO⁺CD45RA⁻CCR7⁻ effector phenotype irrespective of their original differentiation status, thus determining the higher frequency of CCR7⁻CD45RO⁺ T cells observed in Figure 6A at d3.

It is also known that Cy preferentially depletes proliferating cells. As expected³⁵, *in vivo* treatment with high dose of Cy, given at d3 and d4 after transplantation, depleted activated and proliferating cells almost completely at d7

post-haploBMT, leaving a population of relatively quiescent T cells (Figure 6A) and allowing the identification of immune T cell phenotypes preceding the initiation of reconstitution without bias. Given that only donor T cells are responsible for peripheral T cell reconstitution, as shown in Figure 5 and also assessed by the analysis of donor HLA type at the single cell-level (Figure 6B), we extensively analyzed the phenotype and maturation status of recipient and donor T cells by 18-color flow cytometry to identify correlates of persistence *in vivo*. Based on previous data^{72,83}, multiple markers were used simultaneously to accurately identify T cell phenotypes, as indicated in Methods (Chapter 3.4, Figure 3). While recipient cells preferentially expressed a memory phenotype (with some differences between the CD4⁺ and CD8⁺ T cell compartments in terms of T_{CM}, T_{EM} and T_{TE} cell distribution; Figure 6C, D), including activated (HLA-DR⁺) and senescent (CD57⁺) cells⁸⁰ (Figure 6C), donor cells predominantly displayed a T_{SCM} phenotype, characterized by the co-expression of multiple naïve-associated antigens as well as by the T_{SCM} core phenotypic marker CD95^{71,75} (Figure 6C,D). The frequencies of T_{SCM} among the donor populations largely exceeded those observed in the corresponding grafts, both for CD4⁺ and CD8⁺ T cells (Figure 6D), and in the PB of healthy donors (typically 2-3% of total T cells)⁷¹. In the following weeks, T_{SCM} were replaced by more differentiated T_{CM} and T_{EM} cells (Figure 6E). Subsequently, a longitudinal analysis of the absolute counts of CD3⁺, CD4⁺ and CD8⁺ T cells (Figure 6F), and also CD4⁺ and CD8⁺ T cell subsets (

Figure 7), was performed. Rapid increases were observed from week 6 post-haploBMT, when the immunosuppressive drug MMF was discontinued, that were followed by equally rapid declines at week 9. These were possibly caused by the apoptotic loss of highly-activated effector cells, as proposed previously¹²³.

Subsequently, T cells progressively recovered over time (Figure 6F). These dynamics paralleled the rate of proliferation of multiple T cell subsets (Figure 8). During recovery, T cells displayed a predominant T_{TM}, T_{EM} or T_{TE} cell phenotype and tended to normalize only at 1 year post-haploBMT, when CD4⁺ T_{RTE} and CD8⁺ T_N appeared (Figure 6G;

Figure 7). T_{SCM}-phenotype cells are thus highly enriched early after transplantation and might contribute to subsequent T cell recovery.

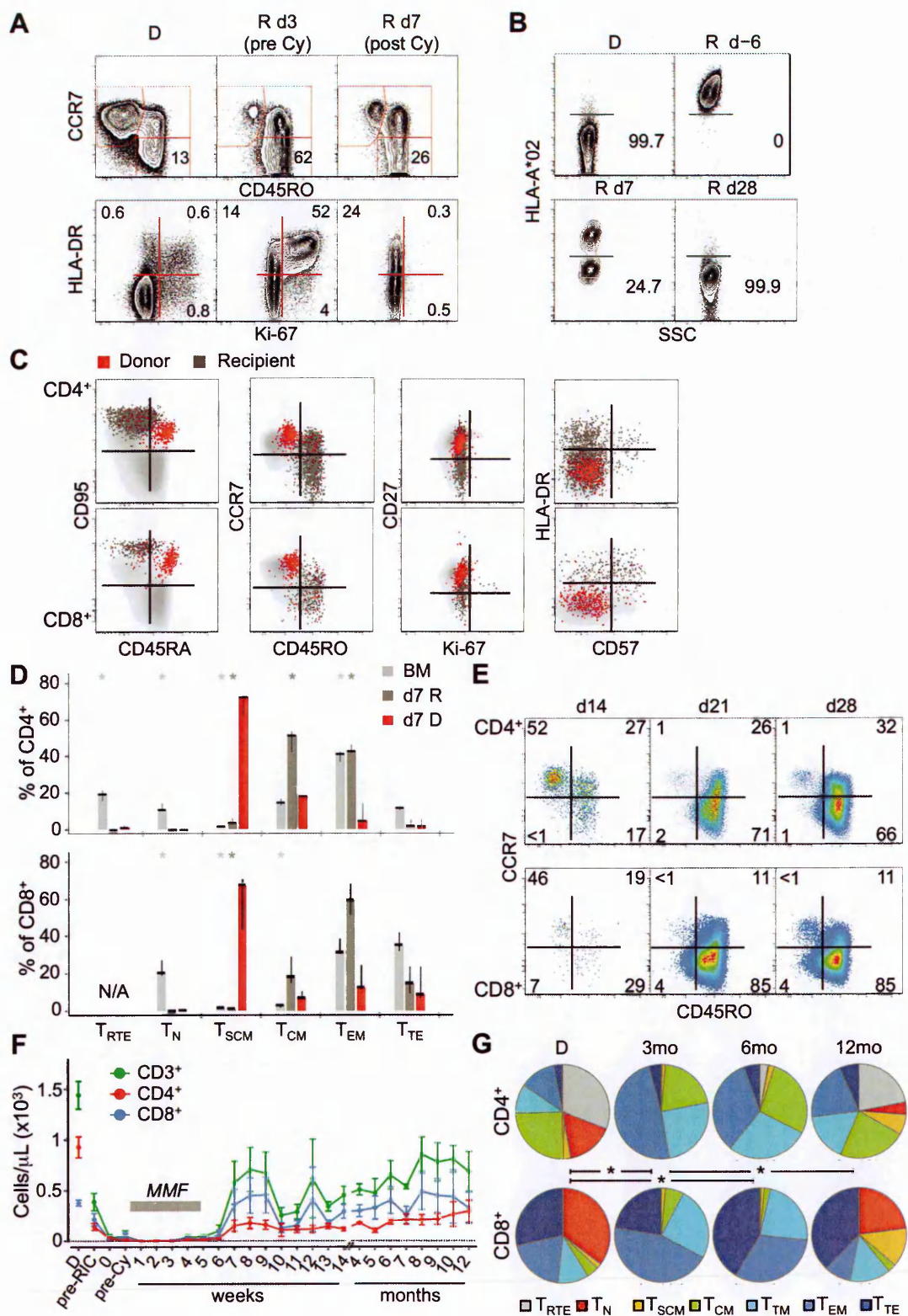


Figure 6: T_{SCM} dominate the T cell compartment before reconstitution. (A) Representative (out of 10) CD45RO/CCR7 (top) and Ki-67/HLA-DR (bottom) expression on PB CD3⁺ T cells in the donor (D) and in recipient (R) at d3 and d7 post-haploBMT. **(B)** Longitudinal identification of donor (D) and recipient (R) CD3⁺ T cells by analysis of the mismatched HLA (in this case HLA-A*02) via flow cytometry. **(C)** Simultaneous analysis of differentiation and

activation markers on PB T cells at d7 post-haploBMT. Donor (D; red) and recipient (R; dark grey) cells are identified by antibody recognizing the mismatched HLA-A*02 (expressed by recipient T cells). Light grey cells in the background are T cells from the PB of a healthy donor. (D) Median±Standard error of the mean (SEM) frequency of D and R T cells (identified as in C) with a given differentiation phenotype in patients at d7 post-haploBMT (n=4). Only donor-recipient pairs whose mismatched HLA could be investigated by flow cytometry are included. *=P<0.05 vs. d7 D cells; Mann-Whitney test. (E) CD45RO/CCR7 expression on T cells at different times post-haploBMT. Numbers in the flow cytometry plots indicate the percentage of cells identified by the gates. (F) Mean±SEM absolute counts of CD3⁺, CD4⁺ and CD8⁺ T cells. (G) Relative median proportion of T cell subsets post-haploBMT. *=P<0.05; permutation test. Details on the number of patients at each time point displayed in F-G are indicated in Figure 2. RIC: reduced intensity conditioning regimen; MMF: mycophenolate mofetil; mo: months; d: day; N/A: Not applicable.

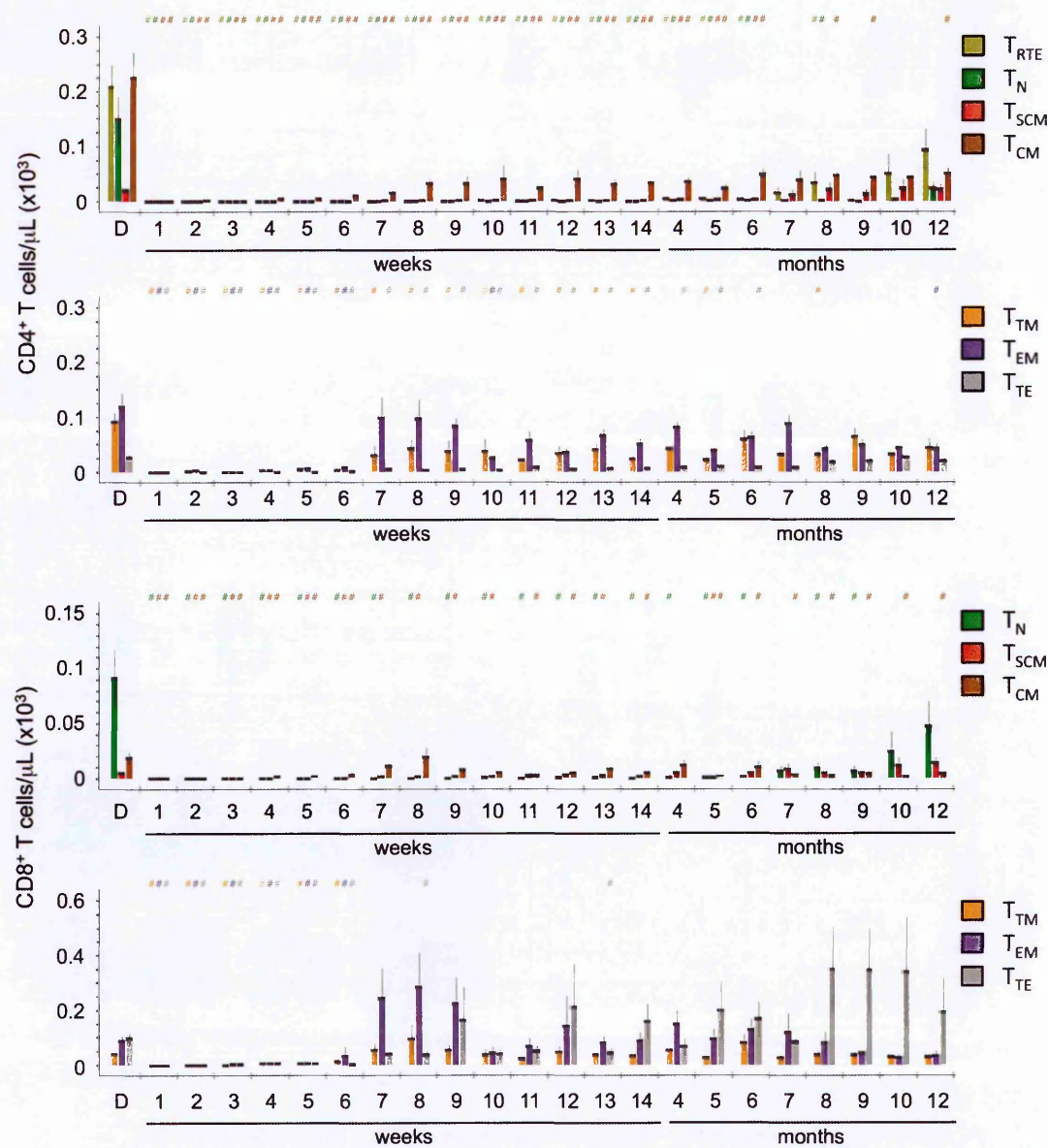


Figure 7: **T cell subsets dynamics following haploBMT.** Mean±SEM absolute counts (cells/ μ L) of T cell subsets from patients following BMT and related donors. The number of patients at each time point is indicated in Figure 2. # = $P < 0.05$ vs. Donor; Mann-Whitney test.

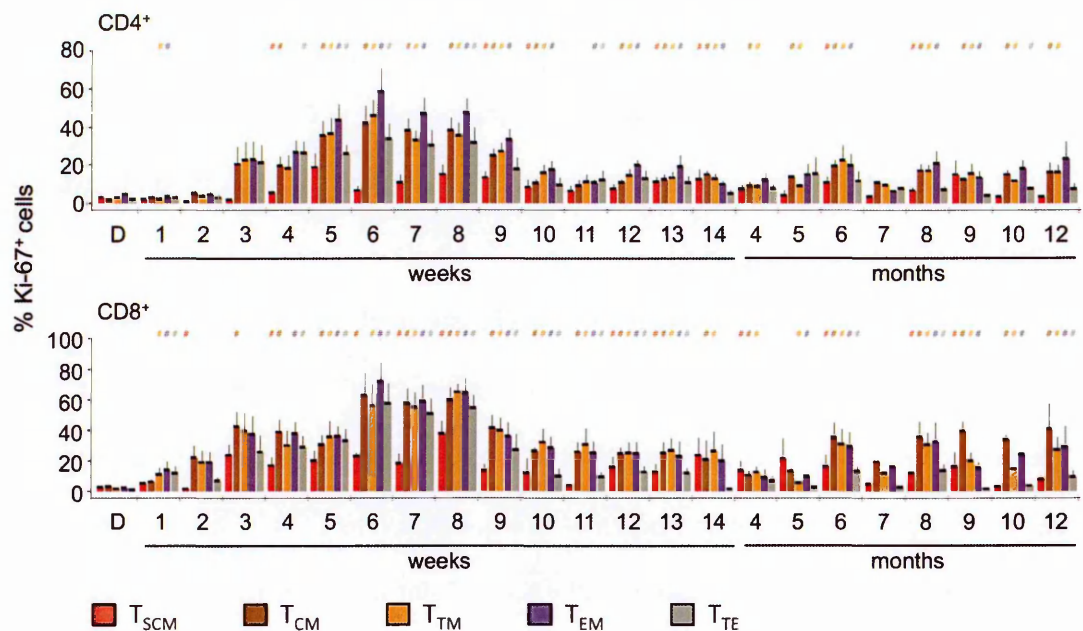


Figure 8: **Proliferation of multiple memory T cell subsets during reconstitution.** Mean±SEM expression of Ki-67 by memory T cell subsets during T cell recovery. T_{RTE} and T_N cells were omitted as no relevant expression of Ki-67 could be detected. Week 5 post-haploBMT corresponds to MMF discontinuation and week 6 to peak T cell proliferation in the majority of the subsets. Details on the number of patients at each time point are indicated in Figure 2. # = $P < 0.05$ vs. Donor; Mann-Whitney test.

4.2.2 Non-alloreactive T_N survival in response to pt-Cy

Given the preferential sensitivity of proliferating cells to Cy, we investigated whether different subsets of T cells have a specific *in vivo* expression of the proliferation marker Ki-67 before Cy treatment (*i.e.*, d3 post-haploBMT). We found that $CD4^+ T_{RTE}$ and both $CD4^+$ and $CD8^+ T_N$ were almost exclusively Ki-67⁻ at this time point, while memory T cells tended to express larger amounts of Ki-67 with progressive differentiation (Figure 9A). This was more evident for $CD4^+$ than for $CD8^+$ T cells. The former also proliferated less compared to $CD8^+$ T cells (Figure 9A). Similar trends were confirmed by gating on donor-derived T cell subsets (Figure 9B). Similar to conventional $CD4^+$ T cells, $CD4^+CD25^{++}CD127^-$ T regulatory

cells (T_{REG}) (Figure 9C) with a naïve phenotype (*i.e.*, $CD45RO^-CCR7^+CD45RA^+$) (Figure 9D,E) preferentially survived Cy treatment (Figure 9D), as recently reported¹⁷², due to their lower proliferation rate compared to memory cells at d3 post-haploBMT (Figure 9F). As a result of increased Ki-67 expression by $CD8^+$ T cell subsets at d3 and the sensitivity of proliferating cells to Cy, the depletion of $CD8^+$ T cells and the increased frequency of $CD4^+$ T cells was observed following Cy treatment *in vivo* (Figure 9G).

It has been proposed that memory T cells are relatively resistant to Cy treatment compared to T_N ²⁵. This is based on mouse studies where allogeneic effector cells were transferred from donor mice following immunization with host cells for 7 days³² or on human studies where unfractionated $CD4^+$ T cells were treated with Cy *in vitro*¹⁷². To better clarify the cellular mechanisms of Cy-mediated depletion of T cells, we set up MLR cultures by using highly-purified naïve or memory T cell subsets and allo-APCs. Auto-APCs were used as a control. T cell activation induces rapid changes in surface phenotypes, including the loss of CD45RA and the up-regulation of CD45RO expression, thereby leading to the hypothesis that the Ki-67⁺ memory fraction observed at d3 (Figure 9A) also contains allogeneic T cells derived from the T_N pool. Incubation of $CD4^+$ T_N or $CD45RO^+$ T_{MEM} with allo-APCs but not auto-APCs for 3 days led to the proliferation (CFSE dilution) and up-regulation of the activation marker CD25 in a fraction of T cells (Figure 10A), indicating that alloreactivity resides in both subsets. CFSE^{low} cells from the T_N pool, originally FACS-sorted as $CD45RO^-$, uniformly up-regulated CD45RO compared to CFSE^{high} cells (Figure 10B). This was even more evident at d5 of culture (Figure 10C,D). Collectively, these results indicate that the Cy-sensitive Ki-67⁺ fraction observed at d3, exclusively identified by memory

phenotypes, contains proliferating cells originating from both the T_N and the memory T cell compartments.

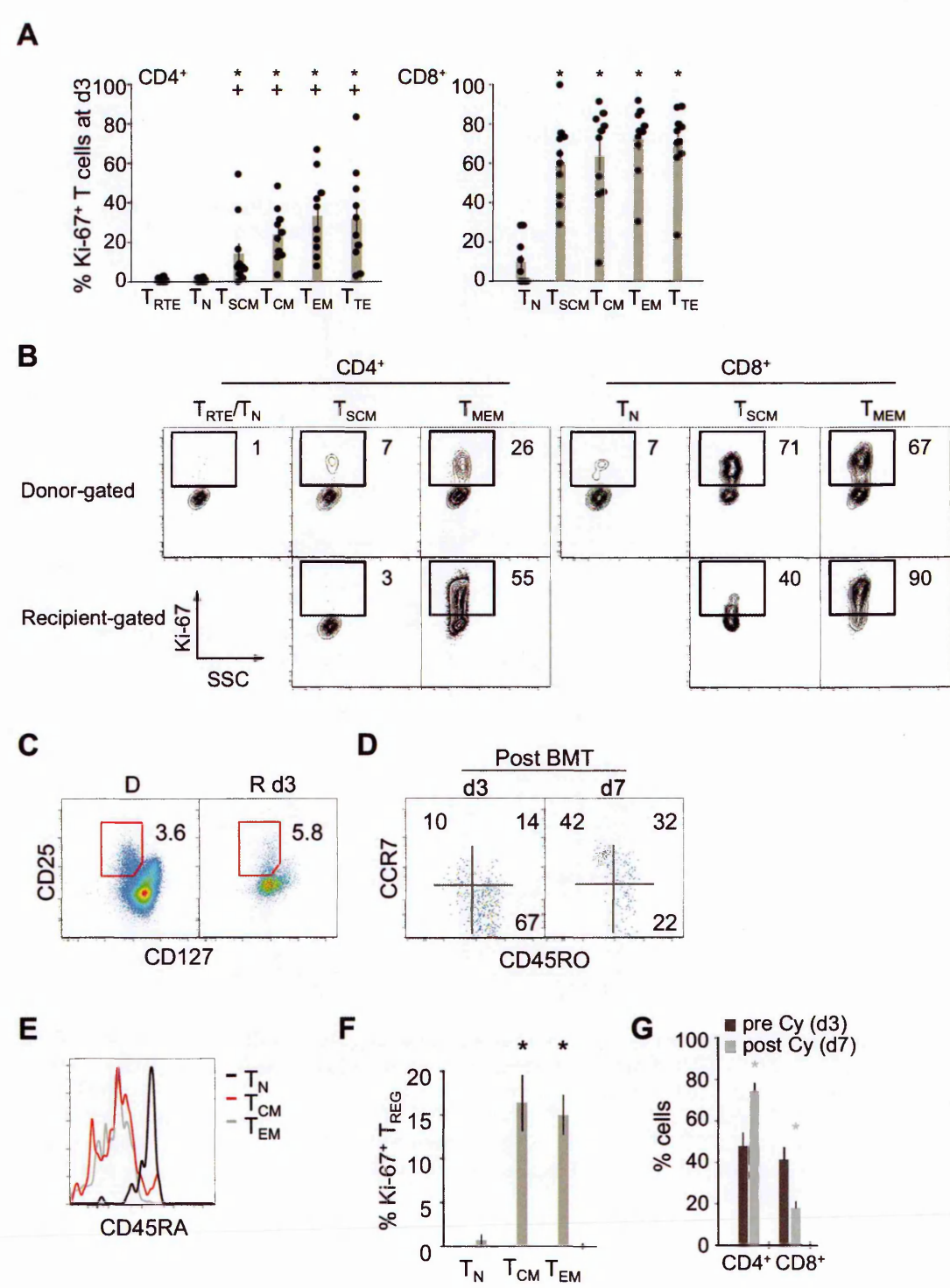


Figure 9: Non-alloreactive T_N preferentially survive pt-Cy. (A) Mean \pm SEM frequency (n=10; each dot represents a patient) of Ki-67 $^{+}$ T cells with a given differentiation phenotype at d3 post-haploBMT. +, *: P<0.05 vs. T_{RTE} and T_N , respectively; Wilcoxon test. (B) Donor and recipient T cell subsets at d3 post-haploBMT identified as in Figure 2 were analyzed for the

expression of Ki-67. T_{MEM} : $CD45RO^+$ T cells. Similar data were obtained from two more patients. **(C)** Identification of $CD4^+$ T_{REG} as $CD25^{++}CD127^-$ in the marrow donor and in the related recipient at d3 post-haploBMT. **(D)** $CD45RO$ and $CCR7$ expression by T_{REG} identified in **C** before (d3) and after (d7) *in vivo* Cy. Note the increase in the proportion of $CD45RO^-CCR7^+$ naïve-like cells. **(E)** Representative $CD45RA$ expression by T_N ($CD45RO^-CCR7^+$), T_{CM} ($CD45RO^+CCR7^+$) and T_{EM} ($CD45RO^+CCR7^-$) T_{REG} cells from a healthy donor. **(F)** Summary of Ki-67 expression in T_{REG} subsets from patients (n=6) at d3 post-haploBMT. *, $P<0.05$ vs. T_N ; Wilcoxon test. **(G)** Mean \pm SEM frequency (n=13) of $CD4^+$ and $CD8^+$ T cells at d3 and d7 post-haploBMT. $P<0.05$ vs. d3; Wilcoxon test. **(B-D)** Numbers indicate the percentage of cells identified by the gates. D: donor; R: recipient.

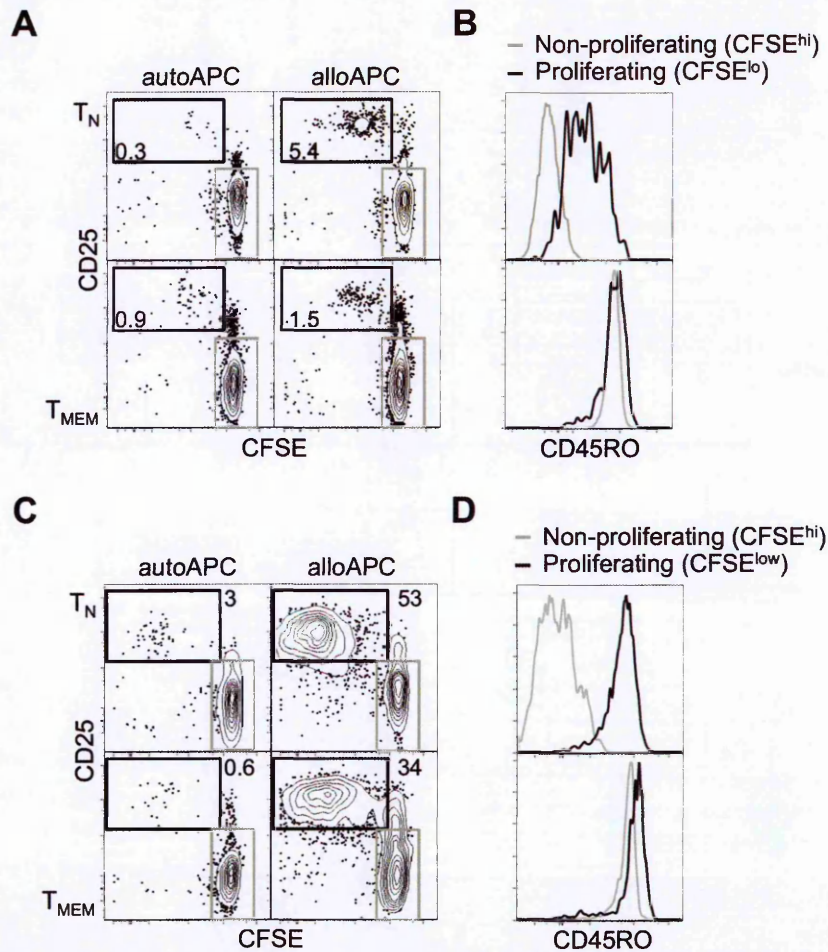


Figure 10: Alloreactive T_N acquire memory phenotypes. **(A)** CFSE dilution and CD25 expression by T_N and T_{MEM} following incubation with auto-APCs or allo-APCs for 3 days. The gate in black identifies $CFSE^{low}$ proliferating cells while that in grey $CFSE^{high}$ non-proliferating cells. **(B)** $CD45RO$ expression by $CFSE^{low}$ and $CFSE^{high}$ T_N (top) and T_{MEM} (bottom) cells identified as in **A**. **(C)** CFSE dilution and CD25 expression following 5 days of incubation with auto-APCs or allo-APCs. **(D)** $CD45RO$ expression by $CFSE^{low}$ and $CFSE^{high}$ T_N (top) and T_{MEM} (bottom) cells identified as in **C**.

4.2.3 T_N -origin of post-transplant T_{SCM}

Given the preferential depletion of $Ki-67^+$ T cells by Cy treatment, it seemed unlikely that the high frequency of T_{SCM} -phenotype cells observed at d7 originated

from the proliferation of a pre-existing T_{SCM} population. Moreover, T cells were still Ki-67⁻ at d7 (Figure 6C), indicating that proliferation did not commence after pt-Cy, at least in the blood. We thus reasoned that T_{SCM} derived from the differentiation of adoptively-transferred T_N . To validate this concept, we followed the expression of CD31, a marker preferentially expressed by early-differentiated $CD4^+ T_{RTE}$ ¹⁷³ and down-regulated in $CD4^+ T_{SCM}$ and T_{MEM} ⁷¹. $CD8^+$ T cells were not studied in this regard, as CD31 has no value in the identification of $CD8^+ T_{RTE}$.

Before transplantation (d0), very few recipient cells could be observed in the $CD45RO^-CCR7^+$ naïve-like gate (NL; *i.e.* comprising both T_N and T_{SCM}), thus obviating the need to differentiate between donor and recipient cells to follow this population of cells (Figure 11A). These NL cells also expressed additional naïve markers, *i.e.*, CD45RA and CD27 (not shown). NL- $CD4^+$ T cells infused with the BM were mostly CD95⁻ but progressively acquired CD95 expression within both the $CD31^+$ and the $CD31^-$ fractions (Figure 11B). This phenotypic shift was independent of pt-Cy, as *in vitro* mafosfamide treatment did not induce CD95 in FACS-sorted T_N (data not shown).

At d7 post-haploBMT, T_{SCM} expressed CD31 at frequencies similar to those of T_N and higher than those of T_{SCM} from the blood of the related donors (Figure 11C), further corroborating the idea that they derived from differentiation of transferred T_N . To exclude the possibility that CD95 up-regulation occurred non-specifically on all T cells, we calculated the fold change in CD95 expression in different $CD4^+$ and $CD8^+$ T cell subsets between d3 and d7, and found that it increased only in NL T cells (Figure 11D). Moreover, *in vitro* incubation of T_N with allo-APCs led to CD95 up-regulation in the non-alloreactive fraction compared to incubation with auto-APCs (Figure 11E,F), thereby indicating that acquisition of the T_{SCM} -phenotype may occur in the allogeneic environment. Such an increase could be prevented by the

addition of anti-MHC class II (for CD4⁺) and class I (for CD8⁺) blocking antibodies (Figure 11E,F).

Alternatively, to test whether d7 T_{SCM}-phenotype cells derived from the proliferation of pre-existing donor T_{SCM} (either CD31⁺ or CD31⁻), we FACS-sorted CD31⁺ T_{RTE} as well as CD31⁻ T_N, T_{SCM} and T_{MEM} (Figure 11G) and incubated them with allo-APCs to monitor CD31 expression in the proliferating (CFSE^{lo}) population. CD31⁺ T_{SCM} could not be tested due to low recovery after FACS-sort. T_{RTE} lost CD31 expression upon CFSE dilution and memory differentiation (Figure 11H). Accordingly, CD31⁻ subsets failed to reacquire CD31 (Figure 11H). Collectively, our results exclude the possibility that d7 T_{SCM} originated from pre-existing T_{SCM} and substantiate their differentiation from adoptively-transferred T_N.

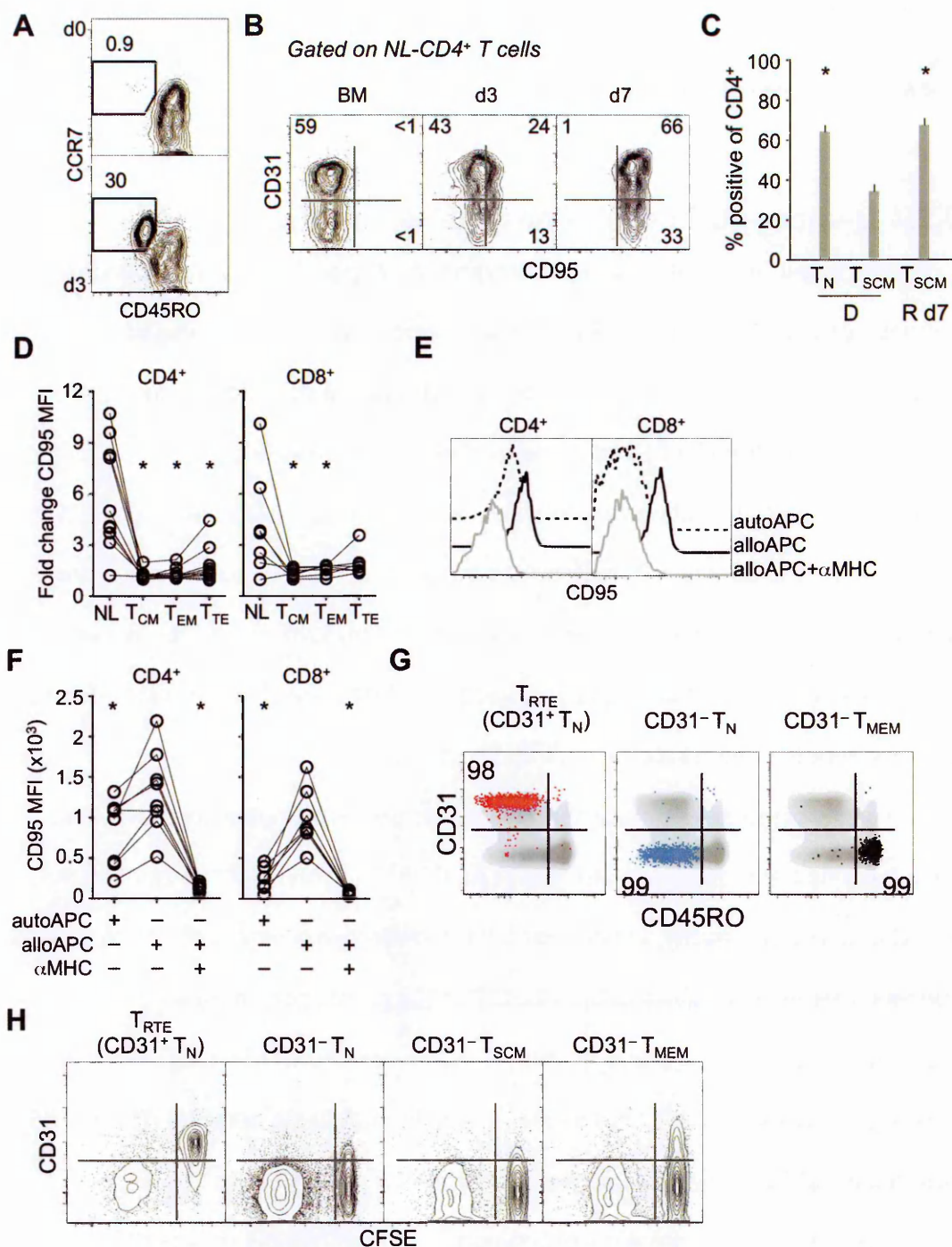


Figure 11: Post-transplant T_{SCM} differentiate from T_N. (A) Representative frequency (out of 12) of CD45RO⁺CCR7⁺ T cells in a patient a d0 and d3 post-haploBMT. (B) Representative (out of 12) CD31 and CD95 expression on naïve-like (NL) CD4⁺ T cells from the BM of a donor and from the PB of the related recipient at different times post-haploBMT. NL: CD45RO⁺CCR7⁺CD45RA⁺CD27⁺. Numbers in the flow cytometry plots indicate the percentage of cells identified by the gates. (C) Mean±SEM CD31 expression on PB CD4⁺ T_N and T_{SCM} from marrow donors (D) and CD4⁺ T_{SCM} from the related recipients (R; n=12) at d7 post-haploBMT. *: P<0.05 vs. D T_{SCM}; Wilcoxon test. (D) Fold change in CD95 median fluorescence intensity (MFI) in different T cell subsets between d3 and d7. *: P<0.05 vs. NL; Wilcoxon test. (E) Representative analysis of CD95 expression by T_N following incubation with different stimuli. (F) Summary of the data obtained as in E (n=8, analyzed in 4 independent experiments; *: P<0.05

vs. allo-APCs, Wilcoxon test). (G) CD45RO and CD31 expression by FACS-sorted T_{RTE} , $CD31^- T_N$ and $CD31^- T_{MEM}$. Around 2,000 sorted cells are shown. Numbers indicate the final purity of the sorted population. (H) CFSE dilution and CD31 expression by FACS-sorted T cell subsets following incubation with allo-APCs for 5 days.

4.2.4 Memory attributes of post-transplant T_{SCM}

The acquisition of the T_{SCM} -phenotype following pt-Cy may have occurred spuriously in T_N . To assess whether T_{SCM} differentiation occurred also at the functional level, we tested the ability of T_{SCM} -phenotype cells from patients to respond to IL-15 (for $CD8^+$) and produce effector cytokines following pharmacological stimulation with PMA/ionomycin (for both $CD4^+$ and $CD8^+$). We first attempted to do this in T cells isolated at d7 or during the early weeks after haploBMT. However, the cells failed to survive *in vitro* following IL-15 stimulation or were unresponsive to PMA/ionomycin treatment (not shown), presumably due to the exposure to immunosuppressive drugs *in vivo*.

To overcome this, we repeated the experiment in samples from later time points, i.e. d41 and d65 after haploBMT. As $CD8^+ T_{SCM}$ could not be purified by FACS due to their paucity, we analyzed the proliferative potential of different T cell subsets after *in vitro* culture of the whole PBMCs population. In previous experiments, purified T_{SCM} and memory T cells were shown to change phenotype only slightly following IL-15 stimulation⁷¹. T_{SCM} from patients between d41 and d65 post-haploBMT diluted CFSE similarly to $CD45RO^+ T_{MEM}$ (Figure 12A,B). Conversely, in line with previous experiments, T_N from healthy donors did not dilute CFSE in response to IL-15 (Figure 12A,B).

To further prove that cells with a T_{SCM} phenotype possess memory attributes, PBMCs from haploBMT patients were stimulated with PMA/ionomycin and their effector cytokine production profile was determined. *In vitro* treatment with these agents did not change the relative proportion of T cell subsets among $CD4^+$ and

CD8⁺ T cells (Figure 12C). T_{SCM} displayed a combination of IFN γ , IL-2 and TNF expression similar to that of naturally occurring T_{SCM}, but distinct from the functional profiles of T_N and T_{MEM} from healthy donors (Figure 12D). This combination is reminiscent of the functionality of T_{SCM} in nonhuman primates⁷³. Collectively, these data show that T_N-derived T_{SCM} acquire T_{SCM} memory properties after BMT *in vivo*.

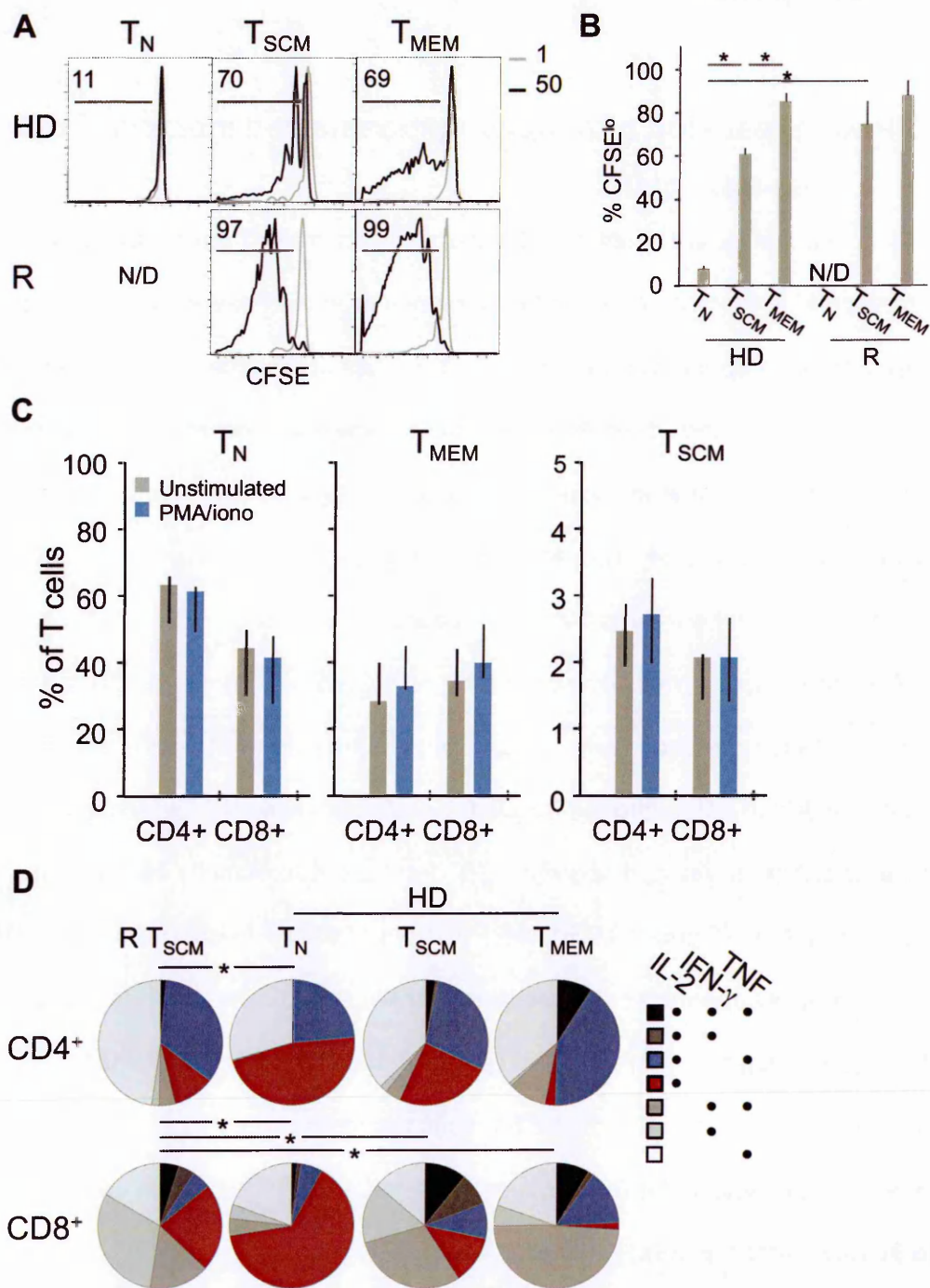


Figure 12: **Post-transplant T_{SCM} possess attributes of naturally occurring T_{SCM} .** (A) Percent CFSE^{low} CD8⁺ T cell subsets from a healthy donor (HD) and a recipient (R) at d41 post-haploBMT after PBMCs culture with 1 ng/mL (grey histogram, serving as a non-proliferating control) or 50 ng/mL (black histogram) IL-15 for 8 days. CD95⁻ T_N were not detected (N/D) in CD8⁺ T cells from Rs *ex vivo* (not shown) or after stimulation with IL-15. (B) Mean \pm SEM CFSE^{low} CD8⁺ T cell subsets (calculated as in A) from HD (n=6) and haploBMT patients. T_{MEM} : CD45RO⁺ memory T cells. * = P<0.05; Mann-Whitney test. (C) Frequency (median \pm SEM) of T_N , T_{MEM} (CD45RO⁺) and T_{SCM} in unstimulated PBMCs from healthy donors (n=4) or after stimulation with PMA/ionomycin for 4 hr. (D) Combinations of intracellular IFN γ , IL-2 and TNF production following PMA/ionomycin stimulation for 4 hr in gated T_{SCM} from patients (n=3; at d35, d42 and d49 post-haploBMT) and in T cell subsets from healthy donors (HD; n=4). * = P<0.05; permutation test.

4.2.5 Poor expansion of adoptively-transferred memory T cells post-transplant

Next, we addressed whether antigen-specific memory T cells may survive post-transplant and expand in the host, either in the presence or in the absence of the cognate antigen. For this purpose, we analyzed the dynamics of multiple antigen-specific T cell populations transferred with the graft. We confined our analysis to ~90 days post-haploBMT to exclude the generation of new T_N by resumed thymic output¹²³. The virtual absence of CD4⁺ T_{RTE} at this time point (

Figure 7) further supports this observation.

We used polychromatic flow cytometry combined with fluorescently-labeled MHC class I tetramers and *in vitro* antigen stimulation followed by intracellular cytokine staining to determine the frequency and phenotype of T cells specific for exogenous antigens. Antigen-specific CD8⁺ T cells, predominantly exhibiting a memory phenotype (Figure 13A,B), were present at similar frequencies in the BM and PB of marrow donors, as demonstrated for Flu and CMV-specific memory CD8⁺ (Figure 13C), thus justifying the use of PB cells when patient BM was unavailable. CD8⁺ T cells specific for Flu epitopes restricted by HLA-A*02 or HLA-A*03 were detectable in the PB of marrow donors but undetectable in the recipients up to 90 days after haploBMT (Figure 14A,C).

Interestingly, CD3⁺ natural killer T (NKT) cells recognizing the α -galactosylceramide analog PBS57 declined as well (Figure 14C). These specific cells, as well CMV-specific T cells, were also quiescent (Ki-67⁻), *i.e.*, not preferentially susceptible to pt-Cy at the time of transfer (Figure 13A,B). Similarly, adoptively transferred CMV-specific CD4⁺ and CD8⁺ T cells from a CMV⁺ donor could not be detected in a CMV⁻ recipient (Figure 14B,C), as previously reported¹⁷⁴. Flu-specific CD4⁺ T cells responding *in vitro* to the seasonal influenza vaccine (Flu^{VAX}) behaved in the same way (Figure 14C). It has been demonstrated that early differentiated memory T cells better persist and expand *in vivo* following adoptive transfer^{175,176}. In our setting, the relative abundance of different memory T cell phenotypes among the transferred specific T cells did not influence the capacity to persist or expand *in vivo* (Figure 14D).

To determine whether the presence of the cognate antigen influenced the expansion of adoptively transferred memory T cells, we studied CMV-specific T cells in the PB of CMV^{+/+} donor/recipient pairs. In these individuals, CMV-specific T cells were detectable in the post-transplant period and were of donor origin, as indicated by analysis of the mismatched HLA-A*02 (Figure 15A,B). FACS-sorting of these cells followed by sequencing of *TCRB* gene rearrangements revealed that some CDR3 sequences overlapped between donors and recipients in most cases at the amino acid (Figure 15C,D) and nucleotide level (data not shown), although the majority appeared to be unique. Overall, these data indicate that adoptively-transferred memory T cells may survive pt-Cy but expand in the recipient only in the presence of their cognate antigen.

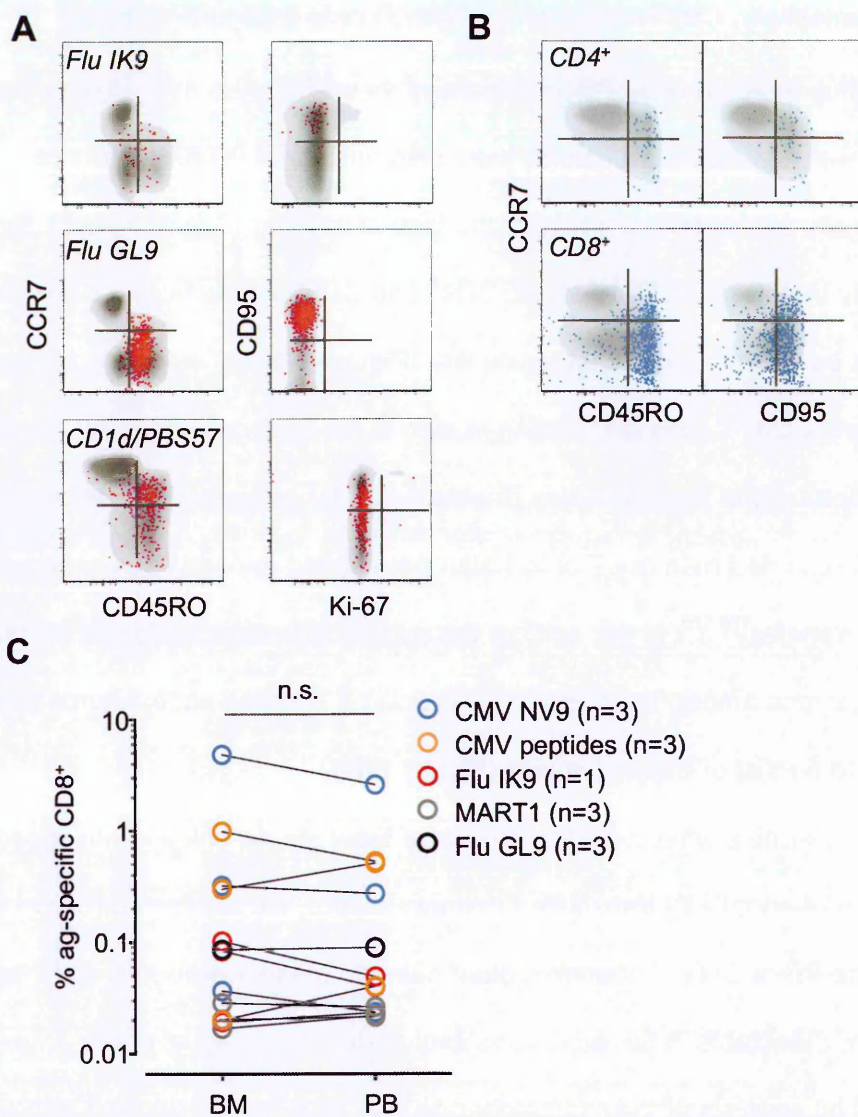


Figure 13: **Phenotype and localization of antigen-specific CD4⁺ and CD8⁺ T cells.** (A) CD45RO, CCR7, CD95 and Ki-67 expression in CD8⁺ T cells specific for Flu IK9 and Flu GL9, and in CD3⁺ NKT cells binding the CD1d/PBS57 tetramer complex. Antigen-specific T cells are depicted as red dots overlaid on total CD8⁺ T cells (grey background). (B) CD45RO, CCR7 and CD95 expression by CMV-specific CD4⁺ and CD8⁺ T cells as identified in Figure 14B. Blue dots are the T cells producing IFN γ following stimulation *in vitro* with the CMV pp65 peptide pool. (C) Frequency of antigen-specific CD8⁺ T and CD3⁺ NKT cells in bone marrow (BM) and peripheral blood (PB) of donors (n=13). n.s.= not significant; Wilcoxon test.

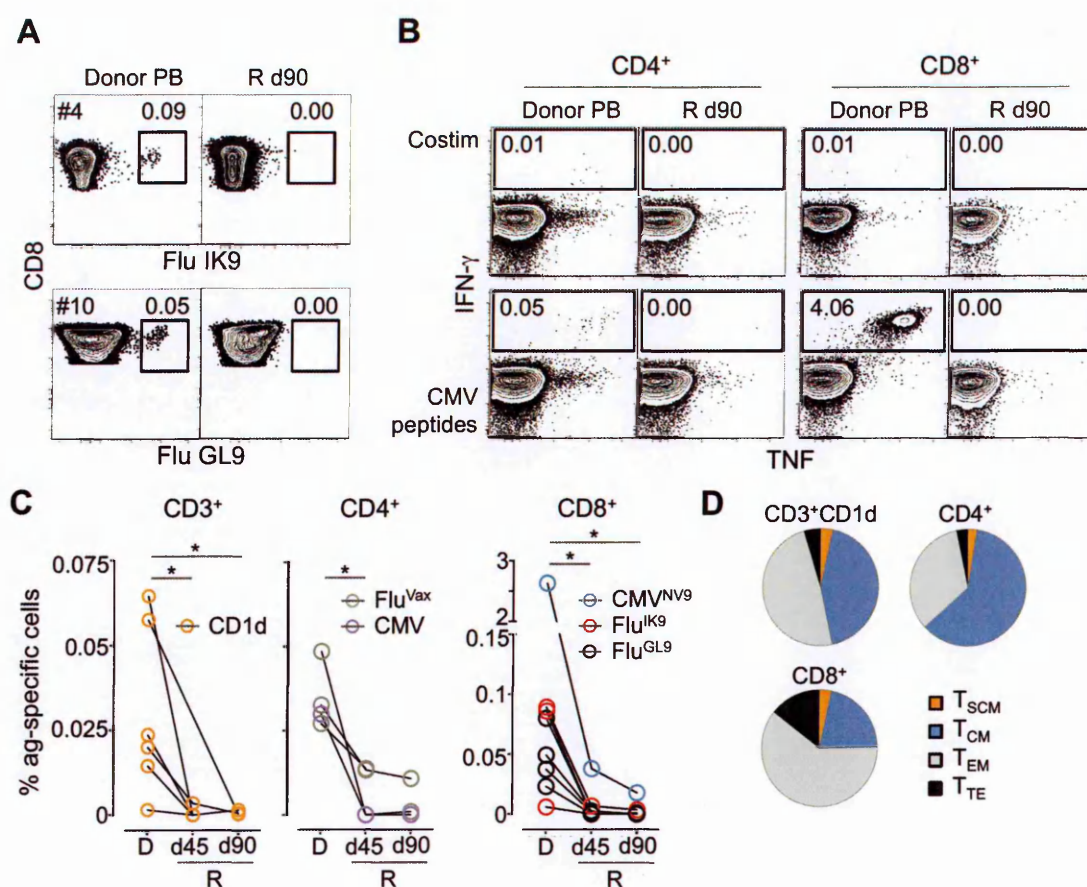


Figure 14: **Adoptively-transferred memory T cells do not expand in the absence of cognate antigen.** (A) MHC class I tetramer identification of CD8⁺ T cells specific for Flu IK9 and Flu GL9 epitopes in the PB of marrow donors (D) and the related recipients (R) at d90 post-haploBMT. (B) TNF and IFN γ production by CD4⁺ and CD8⁺ T cells from haploBMT #10 D/R pair (CMV⁺ and CMV⁻, respectively) following *in vitro* stimulation with the CMV pp65 peptide pool. In both A and B, numbers indicate the percentage of cells identified by the gates. (C) Summary of the frequency of CD3⁺ NKT cells binding CD1d/PBS57 tetramer, and Flu and CMV-specific CD4⁺ and CD8⁺ T cells from the PB of D and the related R at d45 and d90 post-haploBMT. *= $P < 0.05$, Mann-Whitney test. (D) Differentiation phenotypes of the antigen-specific T cells identified in C. Data are presented relative to total memory T cells.

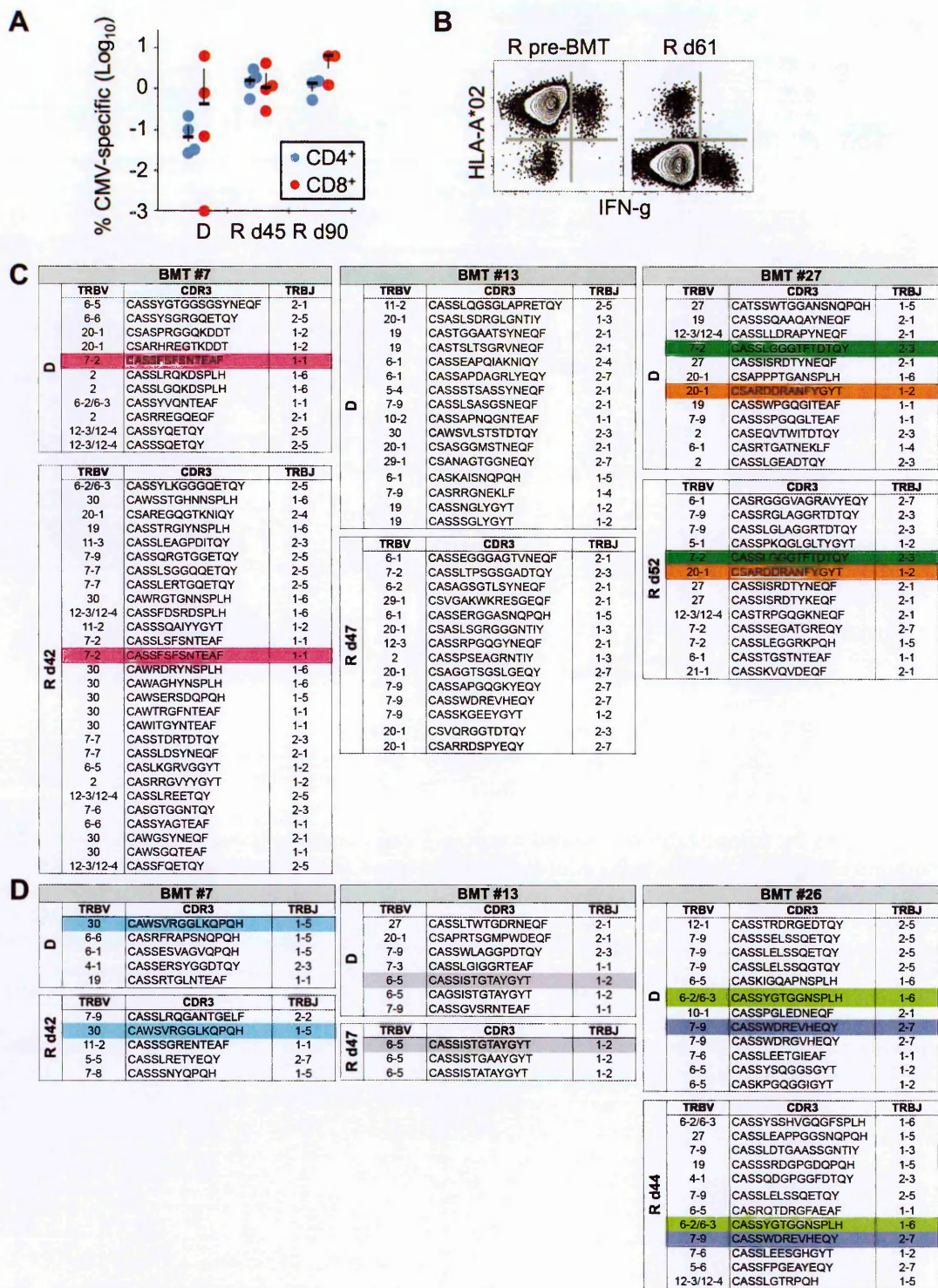


Figure 15: **TCRB** clonotypic analysis of circulating CMV-specific T cells in CMV^{+/+} donor/recipient pairs. (A) Frequency of CMV-specific memory T cells in haploBMT patients and related donors. (B) Representative analysis of donor-derived CMV-specific T cell responses detected by simultaneous analysis of the mismatched HLA (in this case the donor was HLA-A*02^{*}) and intracellular IFN γ following stimulation with CMV pp65 overlapping peptide mix. Plots show Aqua⁺CD3⁺ cells. (C, D) Clonal composition of CMV-specific CD4⁺ (C) and CD8⁺ (D) T cells in CMV^{+/+} donor/recipient pairs. Overlapping sequences are highlighted by colors. D: donor; R: recipient; d, day after haploBMT.

4.2.6 Persistence and memory differentiation of adoptively-transferred T_N

Next, we analyzed whether donor T_N that survived pt-Cy contribute to reconstitution at the antigen-specific level. We followed the fate of self/tumor-specific T cells by using MHC class I tetramers. The staining specificity for MART-1 and WT-1 is shown in Figure 16A. Two CMV⁺ patients receiving a haploidentical graft from CMV⁻ donors were able to mount CMV-specific CD8⁺ T cell responses (Figure 16B), suggesting that activation and depletion of CMV-specific T cells does not occur within 4 days post haploBMT. These CMV-specific responses are thought to originate from the transferred T_N and not from pre-existing memory cells, as CD8⁺ T cells, differently from CD4⁺ T cells¹⁷⁷, were shown to lack pathogen-specific memory T cells in unexposed individuals¹⁷⁸.

T_N specific for self-tumor epitopes, including MART-1 and WT1, behaved similarly. These specific cells are present at relatively high frequencies in PB and BM of healthy individuals (as demonstrated for MART-1 specific cells in Figure 13C) and can thus be detected by MHC class I tetramers^{179,180} (Figure 16A). CD8⁺ T cells specific for MART-1 and WT1 mostly displayed a T_N -phenotype in healthy donors (Figure 16C,E) and were able to persist up to d90 post-BMT (Figure 16D). These cells converted to a CD45RO⁺CCR7⁻CD95⁺ phenotype (as soon as d45 post-haploBMT for MART-1), thus suggesting effector/memory differentiation (Figure 16C,E). On the basis of these data, we conclude that antigen-specific T_N survive pt-Cy and contribute to immune reconstitution in the lymphopenic host.

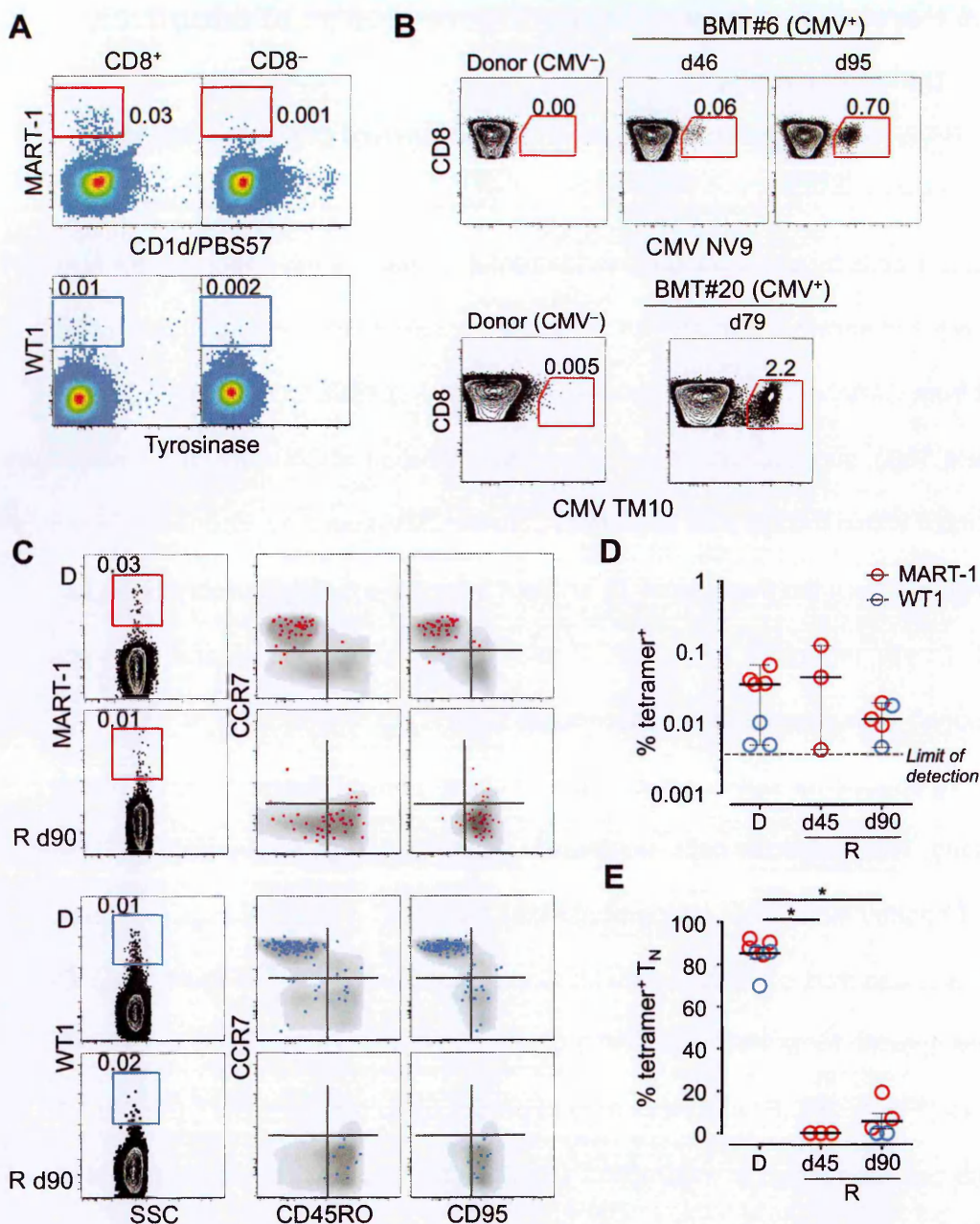


Figure 16: Persistence and memory differentiation of adoptively-transferred T_N. (A) Specificity of tetramer staining in the CD8⁺ and CD8⁻ T cell fractions from healthy HLA-A*02⁺ donors. Note that some cells stain positive for two tetramers and are thus excluded from the analysis. (B) Frequency of CD8⁺ T cells in PBMCs from two CMV⁻ donors and matched CMV⁺ recipients at different time points post-haploBMT. (C) Frequency and phenotype of MART-1 (red dots) and WT1 (blue dots)-specific CD8⁺ T cells identified by MHC class I tetramers. Numbers indicate the percentage of cells in each gate. Tetramer⁺ T cells are overlaid on top of total CD8⁺ T cell populations depicted in grey. In B and C, numbers indicate the percentage of cells identified by the gates. (D) Mean±SEM frequency of the cells identified in B. (E) Frequency of MART-1⁺ and WT1⁺ CD8⁺ T cells with a T_N-phenotype in Ds and Rs at different time points post-haploBMT. * = P < 0.05, Mann-Whitney test.

4.3 B cell reconstitution after transplantation

4.3.1 B cell recovery after haploBMT is donor-dependent

Ten consecutive patients who underwent haploBMT were followed for a median duration of 24 weeks (range: 4-26 weeks) to study the dynamics of B cell reconstitution. Recovery of B cells, defined as $CD3^-CD14^-CD19^+CD20^+$, was assessed by flow cytometry analysis at different time intervals after haploBMT according to the scheme provided in the section "Sample collection" (Figure 2).

B cells could not be detected within the first 4 weeks after haploBMT (data not shown), but started to appear at week 4 or 5 subsequent to the transplant (Figure 17A). At these time points, the absolute counts of PB B cells in the recipients was always <2 cells/ μ L, a number significantly lower compared to that of the marrow donors. Despite the high variability observed among the patients at the investigated time points, the B cell count tended to increase over time starting from week 7 post haploBMT and, in some patients, reached levels similar to that of the healthy donors (Figure 17A). From week 9, we did not detect any significant difference in the B cell absolute count between the transplanted patients and the healthy donors (Figure 17A).

As showed in Figure 5, the donor-recipient chimerism analysis of PB leukocytes and of sorted $CD3^-CD56^-CD14^-CD19^+$ circulating B lymphocytes, revealed that almost all the B cells detected in the recipients derived from the marrow donors (Figure 5). Flow cytometry analysis able to identify HLA haplotypes on $CD20^+$ B cells confirmed the donor origin of the reconstituting PB B cells (Figure 17B).

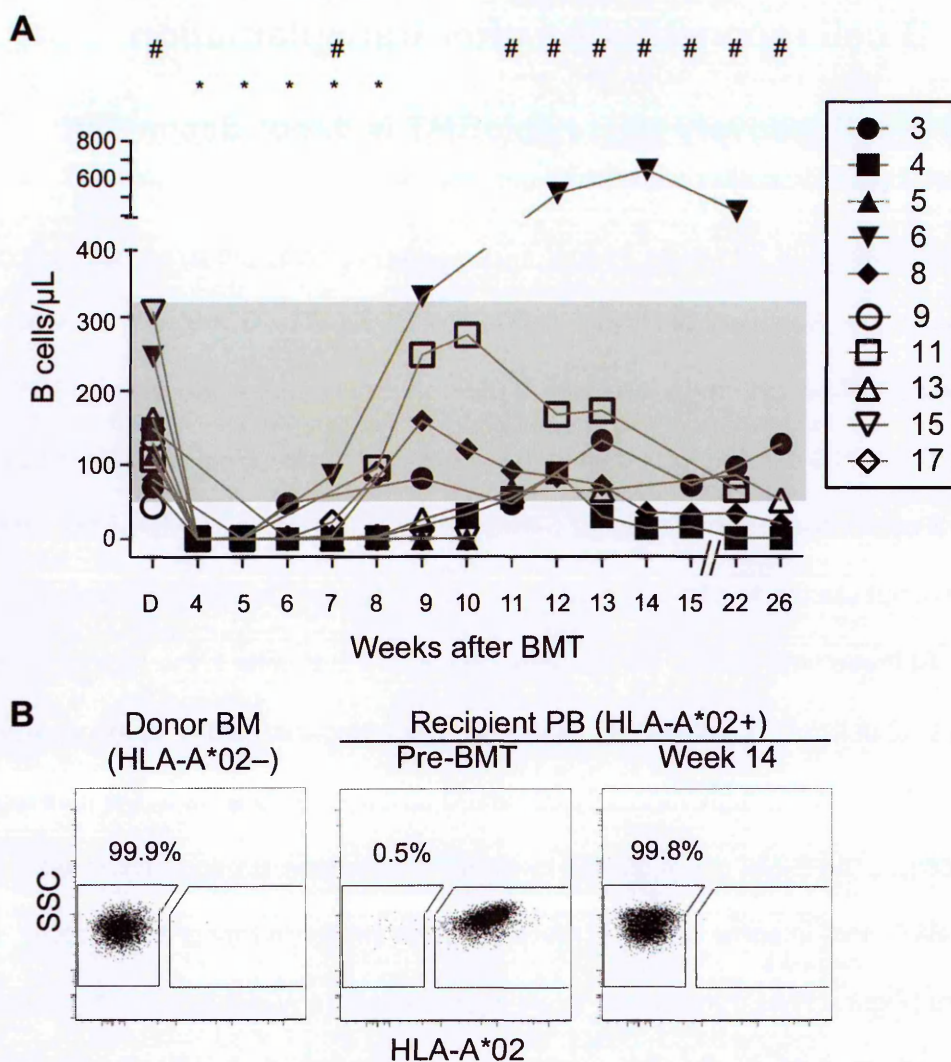


Figure 17: B cell recovery after haploBMT is donor-dependent. (A) Absolute numbers (cells/ μ L) of circulating CD20⁺ B lymphocytes in transplanted patients at different time points after haploBMT compared to those of related BM donors (D). Single donor/recipient pairs are identified by different symbols, as depicted in the legend. Grey box indicates the range of peripheral B cells observed in healthy donors. Asterisks indicate the statistical significant differences between BM recipients and donors, while hash-marks indicate those in the recipients between a given time point and week 4 post haploBMT (*, # = $P < 0.05$; **, ## = $P < 0.01$); Wilcoxon test **(B)** Representative flow cytometry dot plot graphs showing the percentage of expression of HLA-A*02 on circulating B cells in an HLA-A*02⁻ BM donor and in the related HLA-A*02⁺ BM recipient before and 14 weeks after haploBMT. Similar data were obtained from two additional patients.

4.3.2 Reconstituting PB B cells do not proliferate and retain an immature/transitional phenotype in the first weeks after haploBMT

Given that B cells are physiologically present in the BM, that the haploBMT protocol under investigation is based on the infusion of an unmanipulated BM³ and that B cells detected in the recipients derived from the marrow donors, we hypothesized that mature naïve and memory B cells could be adoptively transferred with the graft and thus provide an early source of adaptive immunity in the host. We also reasoned that if B cells are able to persist, they would display a phenotype similar to those found in the BM. However, B cells were barely detectable in the circulation during the first 4 weeks post haploBMT and remained low in absolute counts and relative percentages (as compared to total lymphocytes) until week 8 (Figure 17A and Figure 18A). These data suggest that transferred donor B cells cannot persist in the host.

To determine the phenotypic properties of the recovering B cells, we analyzed the maturation stage of the same cells by using a polychromatic flow cytometric approach able to simultaneously detect multiple B cell differentiation markers. At weeks 5 and 8 after transplant, the vast majority of PB B cells displayed a CD38^{bright}CD10⁺ immature or “transitional” phenotype, whereas the largest fraction of BM-derived B cells showed a CD38^{dim}CD10⁻ mature phenotype (Figure 18B). Indeed, only a small proportion of CD38^{bright}CD10⁺ transitional B cells could be detected in the donor BM (Figure 18B). To exclude the possibility that the BM-derived CD38^{bright}CD10⁺ B cell subset survived post-transplant Cy infusion and underwent homeostatic proliferation in the post-transplant lymphopenic environment¹⁸¹, we evaluated the expression of the proliferation marker Ki-67. Figure 18C shows that transitional B cells at the time of immune recovery (week 8)

were mostly negative for Ki-67 and their rate of proliferation, albeit a little increased, was similar to that observed in PB CD38^{bright}CD10⁺ B cells of the donor.

On the basis of these data, we conclude that no mature B cells survive in the host with the infusion of an unmanipulated BM followed by pt-Cy. Rather, we hypothesize that B cell recovery depends on a new maturation process starting from haematopoietic stem cells/B cell precursors transferred with the BM.

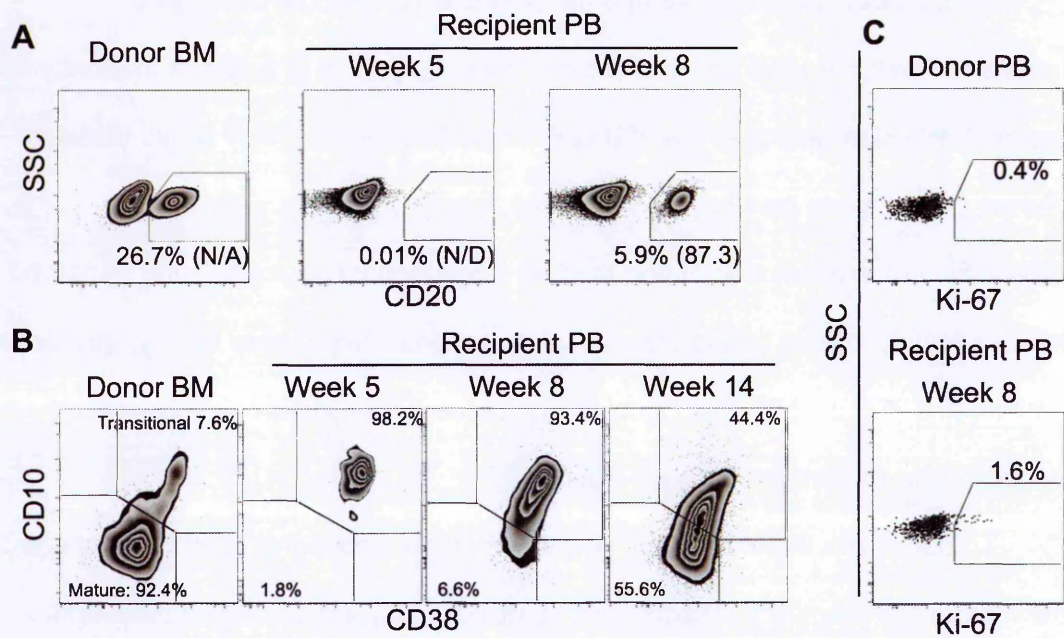


Figure 18: Reconstituting PB B cells do not proliferate and retain an immature/transitional phenotype in the first weeks after haploBMT. (A) Flow cytometry contour graphs showing the frequencies of CD20⁺ B cells in the BM of a representative donor and in the PB of the related recipient early post-BMT (week 5) and at recovery of the B cell compartment (week 8). B lymphocyte recovery is achieved when the B cell count reaches ≥ 50 cells/ μ L. (B) Flow cytometry contour graphs showing the percentage of circulating transitional (CD38^{bright}CD10⁺) and mature (CD38^{dim}CD10⁻) CD20⁺ B cells from a representative BM donor and the related recipient at weeks 5, 8 and 14 after haploBMT. (C) Flow cytometry dot plot graphs showing the percentage of circulating CD20⁺CD38^{bright}CD10⁺ transitional B cell expressing Ki-67 in the PB of a representative BM donor and the related recipient. The absolute number of B cells/ μ L is indicated in brackets (not available (N/A) for the BM of the donor and not detectable (N/D) in the PB of the recipient at 5 weeks after the transplant (A).

4.3.3 B cell recovery in haploBMT recapitulates B cell ontogeny: transitional B cells precede mature B cell appearance

We next analyzed the kinetics of maturation of circulating B cells. As reported in Figure 18B, shortly after haploBMT (week 5), almost all B cells displayed a CD38^{bright}CD10⁺ transitional phenotype, while those in the PB of the donors were mostly mature (median of mature B cells: 3.4% and 92%, respectively; Figure 19A). Starting from week 9, the proportion of CD38^{bright}CD10⁺ transitional B cells progressively decreased (not shown) in favour of an increased proportion of CD38^{dim}CD10⁻ mature B cells (Figure 18B, Figure 19A). To further evaluate the differentiation status of mature B cells, we included markers of naïvety (IgM and IgD) and memory (IgG) in our polychromatic flow cytometry panel and used the gating strategy reported in Figure 4 to identify different subsets of circulating naïve (IgM⁺ and/or IgD⁺) and memory (IgM⁻IgD⁻IgG⁺ and IgM⁻IgD⁻IgG⁻) B cells starting from week 9 after haploBMT, when a sufficient proportion of mature B cells were available for analysis. The vast majority of these cells showed a naïve phenotype for at least 26 weeks after haploBMT (Figure 19B), while only a small proportion of memory B cells (IgG⁺ as well as IgG⁻) were present (Figure 19C). Altogether, these data confirm that B cell recovery depends on a new maturation process starting from B cell precursors transferred with the BM.

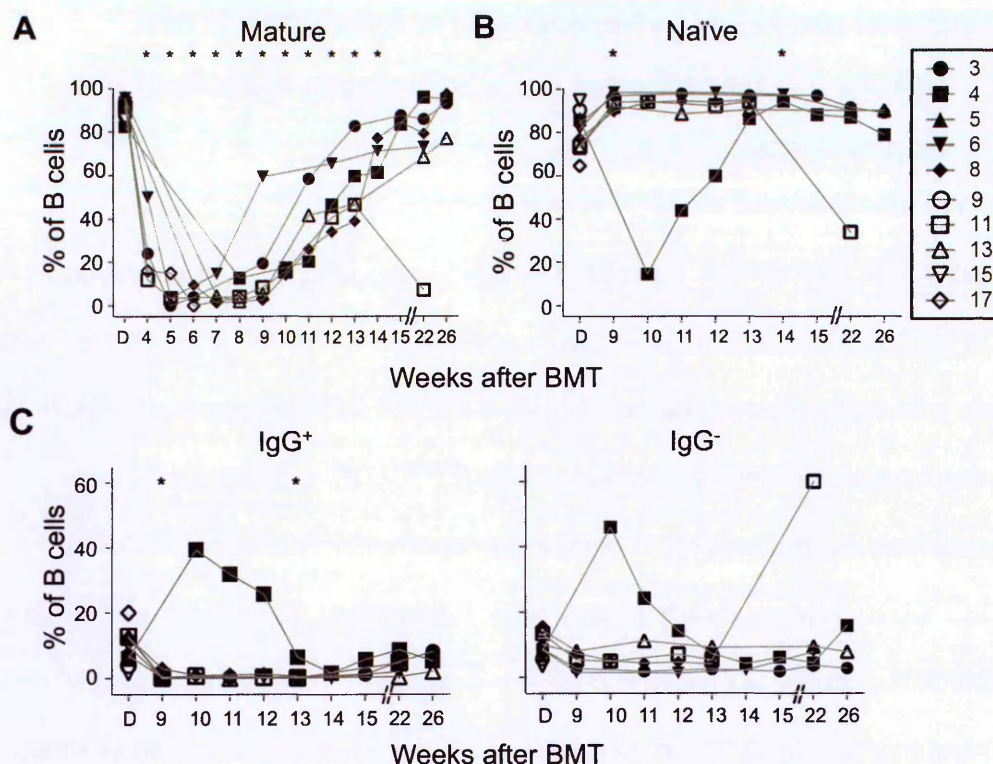


Figure 19: **B cell recovery in haploBMT recapitulates B cell ontogeny: transitional B cells precede mature B cell appearance.** (A) Percentage of CD38^{dim}CD10⁻ mature B cells in the PB of BM donors and related recipients at different time points after haploBMT. Single donor/recipient pairs are identified by different symbols, as depicted in the legend. Asterisks indicate the statistical significant differences between BM recipients and donors (* = P<0.05); Wilcoxon test. (B) Percentage of naïve circulating B cells in BM donors (D) and patients at different time points from week 9 after haploBMT. (C) Percentage of memory IgG⁺ (left graph) and memory IgG⁻ (right graph) circulating B cells at different time points from week 9 after haploBMT. Data are depicted as in (A).

4.3.4 Maturation of transitional B cells in haploBMT requires 4 steps of differentiation

To further investigate the steps of B cell maturation during immune recovery, we analyzed the expression of CD5, a regulator of B cell activation, and CD21, a component of the B cell co-receptor complex, on transitional B cells. These surface markers have been proposed to characterize different stages of CD38^{bright}CD10⁺ transitional B cell development^{131,132}. Similarly to previously reported phenotypic distributions, we observed 3 distinct transitional PB B cell subsets during the course of immune reconstitution following haploBMT: T1 (CD5⁺CD21⁻), T2

(CD5⁺CD21⁺) and the CD5⁻CD21⁺ subset (Figure 20A). In addition, we report an additional stage, here named T0, which is characterized by the absence of CD5 and CD21 and seems to precede the aforementioned T1 and T2 during B cell recovery.

The distribution of patients' transitional B cells among the differentiation stages shortly after BMT was largely different to that present in the PB of the related marrow donors and changed significantly over time. At week 5, transitional B cells mostly displayed a T0 and, in smaller proportion, a T1 phenotype (mean±SEM: 59.4±8.4 and 31.6±7.6, respectively). At week 8, the frequency of T0 cells started to decrease (mean±SEM: 29.7±9.6), whereas the T1 cell subset represented the dominant population (mean±SEM: 41.6±4.1). At later time points (e.g., week 14), the majority of the transitional B cells became CD21⁺, either being T2 or CD5⁻CD21⁺ phenotype cells (mean±SEM: 38.2±11.9 and 21.8±11.6, respectively). The relative distribution of transitional B cell subpopulations at week 14 after haploBMT was similar to that observed in the PB of the related BM donors and showed the following percentages: 14.4±3.9 for T0, 25.4±11.6 for T1, 38.2±11.9 for T2 and 21.8±11.6 for CD5⁻CD21⁺ (Figure 20A).

To verify that the transitional B cell subsets in patients receiving haploBMTs undergo a maturation process leading to the generation of mature naïve B cells, we analyzed the expression of IgD, IgM and CD21 over time. The expression of these surface markers is associated with the acquisition of a fully mature naïve B cell phenotype¹³². Our results show that the levels of CD21, IgD and IgM on transitional B cells increased over time following haploBMT (Figure 20B,C). Of note, the surface expression of IgM at week 15 was significantly higher compared to that of circulating transitional B cells of the related BM donors (Figure 20B,C). These data indicate that, during the course of immune reconstitution following haploBMT,

transitional B cells undergo differentiation and acquire the expression of CD21, IgM and IgD. At approximately 6 months (e.g., week 26) after the transplantation procedure, the amount of CD21, IgD and IgM on the surface of circulating B cells from BM recipients was similar to that of PB B cells of the related donors, thus suggesting that the B cells completed their maturation process.

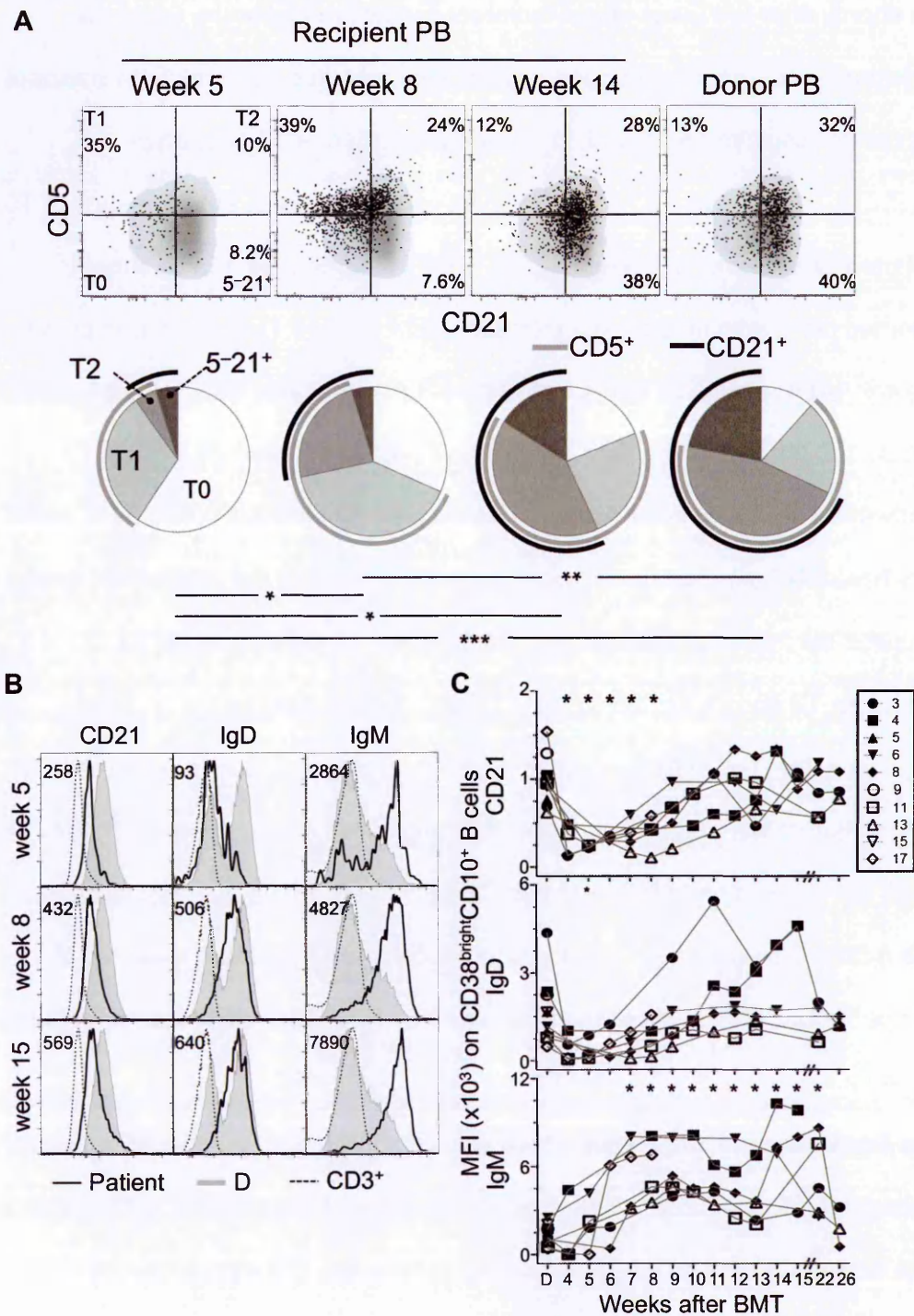


Figure 20: Maturation of transitional B cells in haploBMT requires 4 steps of differentiation. (A) Upper panel: CD5 and CD21 expression on the surface of CD38^{bright}CD10⁺ transitional B cells from a representative BM recipient at weeks 5, 8 and 14 after haploBMT. The expression of the same markers in the peripheral blood of a healthy donor is depicted in gray in the background. Lower panel: pie charts depicting the relative proportions of the 4 subsets identified by CD5 and CD21 expression, as follows: T0 (CD5⁻CD21⁻), T1 (CD5⁺CD21⁻), T2 (CD5⁺CD21⁺) and CD5⁻CD21⁺. * = P<0.05; ** = P<0.01; *** = P<0.001; permutation test. (B) Flow cytometry histograms showing the expression of CD21, IgD and IgM on circulating CD38^{bright}CD10⁺ transitional B cells from a representative BM recipient at weeks 5, 8, 15 after haploBMT (open black line) and from the related donor (filled gray histogram). Dashed histograms indicate the expression of the same markers in CD3⁺ T cells, used as a negative reference. Numbers in the plot indicate the MFI. (C) MFI of CD21, IgD and IgM expression on the same cells from the BM recipients at different time points after haploBMT compared to those of the related BM donors (D). Single donor/recipient pairs are identified by different symbols, as depicted in the legend. Asterisks indicate the statistical significant differences between BM donors (D) and recipients (* = P<0.05); Wilcoxon test.

5 Discussion and conclusions

Understanding the cellular mechanisms underlying immune cell reconstitution following BMT is critical for the development of alternative therapeutic approaches aimed at improving the prognosis of patients affected by hematological malignancies. Such studies also hold the potential to advance our knowledge of immune cell ontogeny in physiological human settings.

Unmanipulated haploBMT with pt-Cy has recently been introduced as an approach that provides a donor for nearly all patients lacking an HLA-matched donor and to improve immune reconstitution through the adoptive transfer of mature immune cells. A comprehensive analysis of immune recovery immediately after transplantation has never been reported in the context of this clinical procedure. Therefore, it is unclear how pt-Cy works as regards the transfer of immunity from donor to recipient. The present study informs the mechanisms of adaptive immune cell reconstitution and focused on T and B cell recovery. Figure 21 summarizes the proposed mechanisms of T and B cell immune reconstitution that arise from this work.

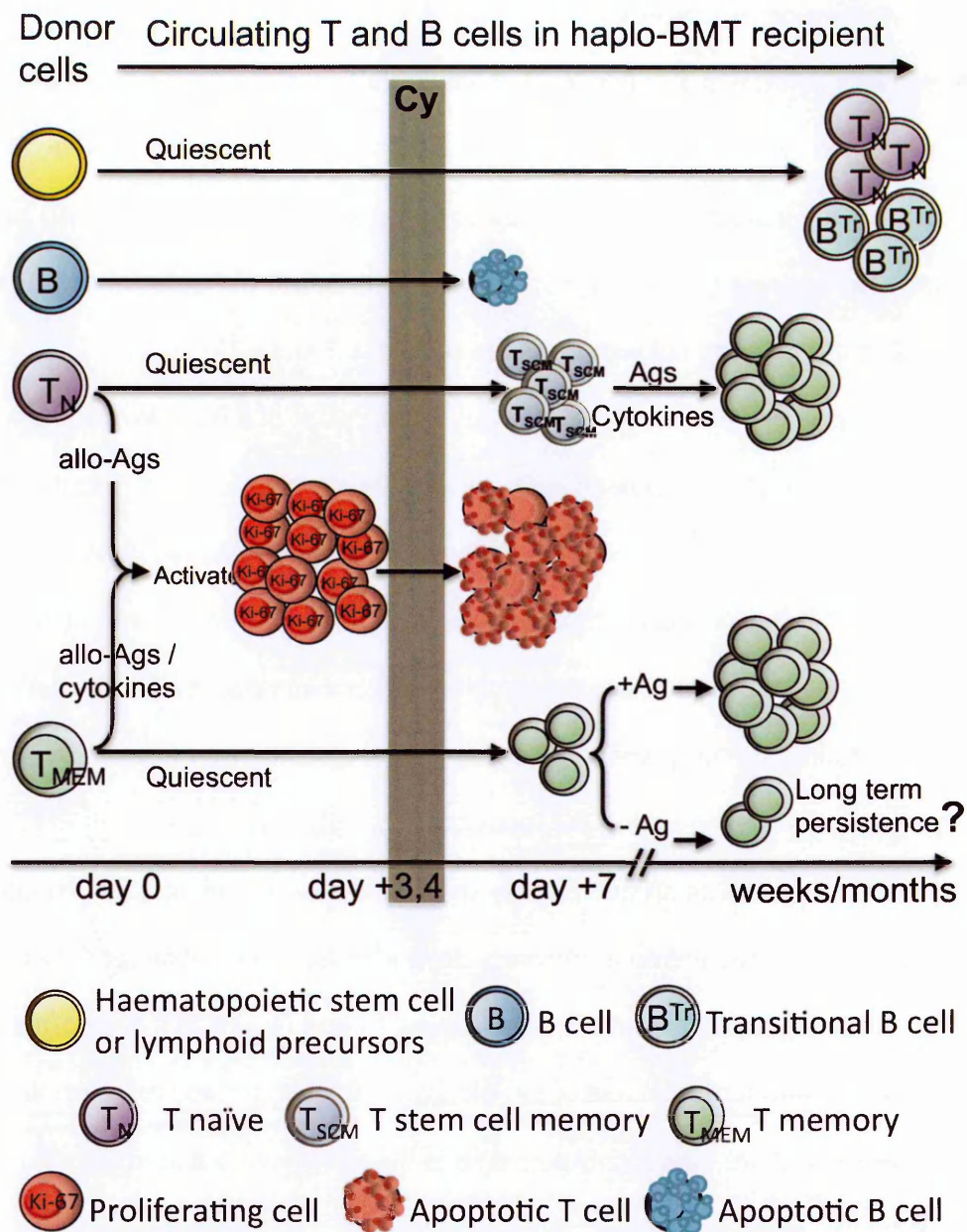


Figure 21: Proposed mechanism for immune reconstitution following haploBMT with non-myeloablative conditioning regimen and high-dose Cy after transplant. Donor BM-derived haematopoietic stem cells, lymphoid precursors, B cells, T_N and T_{MEM} are infused in the recipient with the graft. Haematopoietic stem cells and lymphoid precursors survive Cy given at d3 and d4 after BMT and contribute to B and T cell reconstitution months after BMT, generating T_N and Transitional B cells (B^{Tr}). Conversely, donor BM-derived B cells do not survive after Cy. The allogeneic antigens (allo-Ags) and expansions driven by homeostatic cytokines (e.g., IL-7 and IL-15), the availability of which increases after chemotherapy, induce T cell activation. Activated T cells start to proliferate and uniformly acquire an effector phenotype (HLA-DR+, Ki-67+), irrespective of their origin. Cy treatment preferentially depletes proliferating cells, leaving quiescent T cells. T_N spared by Cy rapidly differentiate into T_{SCMT} as soon as d7 after transplantation. In the following weeks, naïve-derived T_{SCMT} are able to expand in response to self/tumor antigens (ags) or homeostatic expansion, thus generating T_{MEM}. On the other hand, donor BM-derived T_{MEM}, which have survived Cy, are limited in terms of immune reconstitution capacity as suggested by their lower frequency at d7 compared to T_{SCMT}. In the following weeks, the T_{MEM} expansion is dependent on cognate-ag encounter.

The immune reconstitution dynamics of T cells is markedly different from B cell recovery dynamics. Despite the fact that both B and T cells are transferred with the graft in the recipient, only mature T cells are detectable in the recipient after transplant, thus indicating that they survive pt-Cy and provide a source of T cells in transplanted patients (Figure 21). Conversely, B cells are not detectable in the PB, suggesting that they do not survive in the recipient (Figure 21).

We hypothesized that B cell recovery is the result of a *de novo* differentiation process of donor haematopoietic stem cells or lymphoid precursors (Figure 21). The following experimental evidence supports our hypothesis. We observed that CD19⁺CD20⁺ B cells found in PB and bearing an immature/transitional phenotype (*i.e.*, CD38^{bright}CD10⁺) dominate the circulating B cell compartment in the BM recipients during the early weeks following the transplant (Figure 18). This is in line with previous reports analyzing the recovery of B cells following various BMT protocols^{133,142,182}. Although not formally tested because of the very low number of mononuclear cells recovered in the early days after transplantation, pt-Cy likely mediates the loss of transferred B cells (Figure 18 and Figure 21). B cells are in fact known to harbour low levels of the aldehyde dehydrogenase responsible for Cy detoxification and are thus highly sensitive to the drug *in vitro* and *in vivo*¹⁸³.

We took advantage of the complete depletion of B cells in transplanted patients to study the process of B cell differentiation. We performed a detailed longitudinal phenotypic analysis of markers associated with B cell differentiation. The B cell compartment is initially characterized by the presence of transitional B cells and is progressively repopulated by mature naïve B cells that acquire IgG⁻ and IgG⁺ memory phenotypes later on. At the same time, the frequency of transitional B cells gradually decreases (Figure 19). Moreover, the differentiation of transitional B cells is characterized by a process of progressive maturation

identified by CD5 and CD21 expression, as previously suggested by other reports^{131,132}. In humans, CD5 is expressed by immature/transitional B cells and is down-regulated by mature naïve B cells¹²⁹, while CD21 has been proposed to distinguish the CD21⁻ T1 transitional stage of differentiation from the CD21⁺ T2 stage^{131,132}. T1 cells are considered precursors of T2 B cells on the basis of multiple phenotypic and functional aspects¹³².

Recently, it has been proposed that an additional subset of B cells that are phenotypically naïve but retain CD5 expression represents pre-naïve B cells¹³¹. Others referred to cells with a similar naïve phenotype and functional capacity, but negative for CD5, as T3 transitional B cells¹⁸⁴. Our experimental approach allowed us to identify the above-mentioned T1 and T2 stages as well as a CD5⁻CD21⁺ transitional subpopulation. In addition, we report a new putative transitional B cell stage, here named T0, defined by the absence of both CD5 and CD21 (Figure 20).

Discrete transitional B cell subsets develop at different time points following transplantation, thus revealing new insights into the process of B cell maturation in the periphery. Peripheral B cell development during reconstitution begins with the T0 (CD5⁻CD21⁻) subset and culminates with the expansion of CD5⁻CD21⁺ cells as well as CD38^{dim}CD10⁻ naïve B cells at ~6 months post haploBMT. T0 cells represent only a small proportion of transitional B cells in the PB of healthy donors but constitute up to 60% of total CD19⁺CD20⁺ B cells in the patients at 5 weeks post haploBMT (Figure 20).

The recovery of B cells in transplanted patients suggests that B cell differentiation proceeds according to the relationship T0 → T1 → T2 → CD5⁻CD21⁺ transitional → naïve lymphocytes. In accordance with the linear differentiation model that has been proposed for other lymphocyte populations, e.g. T cells^{75,185}, discrete subsets of B cells lose or acquire specific functions with progressive

differentiation. It is unclear at this time whether CD5⁻CD21⁺ transitional and T3 pre-naïve B cells (expressing CD5 or not) constitute the same subset on a functional basis or whether they are related to each other in a precursor-progeny relationship. It also remains to be determined whether T0 cells precede the other subsets of transitional B cells in terms of functional capacity.

At ~6 months post haploBMT, the distribution of PB B cells among the various subsets of differentiation is similar to that observed for circulating B cells from the related BM donors. A slightly reduced proportion of IgG⁺ and IgG⁻ memory cells is observed in transplanted patients versus controls. Patients receiving allogeneic transplants are routinely vaccinated for multiple pathogens starting at 6 months after transplantation. These vaccines would potentially induce antibody responses that are similar to those observed in other transplantation settings, but it is well documented that transplanted patients respond poorly to vaccines for multiple reasons, including the immature characteristics of the B cell compartment¹⁸⁶, the contraction of the B and T cell repertoire and the lack of sufficient T cell help due to the low number of CD4⁺ T cells^{121,187}.

We also report the cellular mechanisms responsible for T cell reconstitution following haploHSCT and pt-Cy at the antigen-specific and clonal level and suggest that "T_N-derived T_{SCM}" play a non-redundant role in this regard. Extensive phenotypic and functional analysis of T cell subsets during the first few days following transplantation reveal that non-alloreactive T_N are spared by pt-Cy because of their delayed activation kinetics. Unexpectedly, T_N are outnumbered by T_{SCM}-phenotype cells at d7, which later display T_{SCM} functional properties. Conversely, as much as 70% of memory/effector-phenotype T cells are proliferating by d3 post-haploBMT and are subsequently depleted by pt-Cy (Figure 21). Experiments performed in mouse models by Mayumi *et al.* showed that pt-Cy

failed to block GVHD following transfer of allogeneic donor splenocytes. The transferred splenocytes were derived from mice that had been immunized with host T cells 7 days before²⁸, thus leading to the hypothesis that memory T cells are more resistant to pt-Cy compared to T_N ²⁵. However, since d7 coincides with the effector phase (*i.e.*, the peak of T cell expansion), escape from pt-Cy possibly occurred because of the relative abundance of transferred effectors rather than the acquisition of memory capacity. Our experiments show that both T_N and memory T cells divide in response to allogeneic stimulation and that T_N rapidly acquire CD45RO, thus indicating that the proliferating memory/effector-phenotype fraction observed at d3 includes T cells derived from both compartments (Figure 6).

T_{SCM} -phenotype cells are the dominant donor T cells in the PB following pt-Cy and are thought to derive from donor T_N . A weakness of the current study is that this relationship could not be demonstrated directly at the antigen-specific or clonal level due to the low abundance of these cells *in vivo*. Nevertheless, the results with polyclonal T cell populations (experiments shown in Figure 11) support the concept that T_N develop into T_{SCM} cells. Although only circulating T cells could be tested for ethical reasons, proliferation of pre-existing donor T_{SCM} or redistribution of T_{SCM} from lymphoid tissues seem unlikely, given the depletion of proliferating cells by pt-Cy and the virtual absence of T_{SCM} in the BM, respectively. Moreover, IL-7 and IL-15, which are elevated in lymphopenic individuals, favours the generation of T_{SCM} from T_N precursors¹⁸⁸. Based on this and the results from the current study, it is concluded that post-transplant T_{SCM} in haploHSCT derive from T_N .

The acquisition of memory/effector phenotypes by self/tumor-specific T cells also suggests that transferred T_N progress through an early T_{SCM} stage (Figure 21). Self-specific memory T cells are rarely observed, unless in pathological conditions. For instance, MART-1-specific memory cells are abundant in metastatic melanoma

but rare in healthy donors^{71,189}, hence leading to the supposition that high antigen load, co-stimulation and local inflammation are required for MART-1-specific T cell priming¹⁹⁰. Alternatively, the conversion of T_N into memory-like cells has been reported in mice to occur in response to lymphopenia (*i.e.*, increased levels of homeostatic cytokines)¹⁹¹⁻¹⁹⁴. Whether homeostatic proliferation rather than cognate antigen stimulation is the major mechanism driving the differentiation of self/tumor specific T_N will require further studies.

Although still present after Cy treatment, residual memory T cells, including T_{CM}, display limited reconstitution capacity, at least in the circulation (Figure 14). The presence of cognate antigen in the post-transplant environment seems to dictate the expansion of antigen-specific memory T cells, as (some) transferred antigen-specific memory T cell clones could be detected in CMV infection-matched transplants (Figure 15). These data, together with those obtained from CMV^{-/+} transplants, implicate that reactivation of CMV and, possibly, other persistent pathogens occurs late enough to allow pathogen-specific T cells to escape pt-Cy-mediated depletion. However, at the same time, the depletion of some non-alloreactive memory T cell clones by pt-Cy cannot be completely ruled out. This might be caused by their more rapid homeostatic proliferation (and subsequent apoptosis) *in vitro*⁷¹ and *in vivo*¹⁰⁴ compared to T_N. Whether T cell memory can persist in these individuals in the relative absence of cognate antigen in body sites other than the circulation remains an open question.

Collectively, our results shed light on the mechanisms governing pt-Cy function *in vivo*, suggesting that transferred T_N may acquire T_{SCM} traits in the lymphopenic environment and subsequently contribute to immune reconstitution.

At the antigen-specific level, pt-Cy allows the generation of primary and memory T cell responses in the presence of persistent antigen in the host.

However, although still present, memory CD4 and CD8 T cells displayed limited expansion capacity in the blood of recipients.

On the basis of the reported data and evidence that the abundance of T_N in BM correlates with improved overall survival and decreased acute GVHD¹⁹⁵, we suggest that the adoptive transfer of large numbers of T_N before pt-Cy may favour the generation of T_{SCM} and boost immune reconstitution.

6 Abbreviations

Allo-APCs	MHC-mismatched antigen-presenting cells
APCs	Antigen-presenting cells
Auto-APCs	Autologous antigen-presenting cells
BMT	Bone marrow transplantation
BM	Bone marrow
CCR	CC chemokine receptor
CFSE	5-(and 6)-carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalovirus
Cy	Cyclophosphamide
d	Day
DCs	Dendritic cells
FK	Tacrolimus
γ c	Gamma chain
GVHD	Graft-versus-host disease
HaploBMT	Haploidentical bone marrow transplantation
HaploHSCT	Haploidentical haematopoietic stem cell transplantation
HLA	Human leukocyte antigen
HSCT	Haematopoietic stem cell transplantation
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
mAb	Monoclonal antibody
MART-1	Melanoma antigen recognized by T cells 1
MFI	Median fluorescence intensity

MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
MZ	Marginal zone
NK	Natural killer
NKT	Natural killer T
NL	Naïve-like
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
pMHC	Peptide-MHC
pt-Cy	Post-transplant infusion of cyclophosphamide
RT	Room temperature
SEM	Standard error of the mean
T _{CM}	Central memory T cells
TCR	T cell receptor
T _{EM}	Effector memory T cell
T _{MEM}	Memory T cells
T _N	Naïve T cells
T _{REG}	Regulatory T cells
T _{RTE}	Recent thymic emigrant T cells
T _{SCM}	T memory stem cells
T _{TE}	Terminal effector memory T cells
T _{TM}	Transitional memory T cells
WT1	Wilms' Tumor 1

7 Bibliography

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8 Publication record

1. Hudspeth, K., Fogli, M., Correia, D.V., Mikulak, J., **Roberto, A.**, Della Bella, S., Silva-Santos, B., and Mavilio, D. (2012). Engagement of NKp30 on Vdelta1 T cells induces the production of CCL3, CCL4, and CCL5 and suppresses HIV-1 replication. *Blood* 119, 4013-4016.
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- 5.* Lugli, E., Gattinoni, L., **Roberto, A.**, Mavilio, D., Price, D.A., Restifo, N.P., and Roederer, M. (2013). Identification, isolation and in vitro expansion of human and nonhuman primate T stem cell memory cells. *Nat Protoc* 8, 33-42.
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9.* **Roberto, A.**, Castagna, L., Zanon, V., Bramati, S., Crocchiolo, R., McLaren, J.E., Gandolfi, S., Tentorio, P., Sarina, B., Timofeeva, I., Santoro, A., Carlo-Stella, C., Bruno, B., Carniti, C., Corradini, P., Gostick, E., Ladell, K., Price, D.A., Roederer, M., Mavilio, D. and Lugli, E. (2015) Role of naïve-derived T memory stem cells in T cell reconstitution following allogeneic transplantation. *Blood* 125, 2855-2864

* Publication related to the current thesis

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